ROLE OF AMMONIUM IONS ON THE MODULATION OF APOPTOSIS IN GLUTAMINE-STARVED CELLS

By

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ABSTRACT

Highly proliferating cells reprogram their metabolism in order to fulfill their requirements in biosynthetic precursors. One consequence of this metabolic adaptation is glutamine (Gln) addiction, a phenomenon whereby cells become dependent on Gln for their survival. How cells sense Gln levels and link this information to the survival machinery remains to be fully understood. Our laboratory previously showed that when Sp2/0-Ag14 (Sp2/0) hybridoma cells were deprived of Gln, apoptosis is triggered within minutes. In this study, I used the Sp2/0 cell line to characterize the role which ammonium ions, a product of glutaminolysis, play in the modulation of cell survival. In a first series of experiments, I demonstrated that, when supplemented with ammonium salts, Gln-deprived Sp2/0 cell cultures showed a significant increase in viability. This effect of ammonium salts was independent of the cells' ability to synthesize Gln, and was not affected by co-treatment with α-ketoglutarate. In agreement with their effect on cell viability, ammonium salts caused a significant reduction in the number of Gln-starved Sp2/0 cells with apoptotic nuclear condensation and fragmentation. Unexpectedly, supplementing Gln-starved Sp2/0 cells with ammonium salts did not alter cytosolic cytochrome c release, caspase-3 activation, DNA fragmentation or nuclear lamin A/C cleavage, indicating that the mechanisms triggering the apoptotic machinery remained intact. In a second set of experiments, I used the Gln antagonists 6-Diazo-5-oxo-L-Norleucine (DON) and azaserine (AZA) to interfere with Gln metabolism in lieu of Gln deprivation. Both antagonists were toxic to Sp2/0 cells, an effect which was attenuated by supplementation with ammonium salts. This indicated that Gln

metabolism was required for cell viability, and that the effect of ammonium salts did not require metabolic pathways utilizing Gln. Unexpectedly, the supplementation of DON or AZA to Gln-starved cultured led to a small but significant increase in cell viability. Moreover, the supplementation with ammonium salts of Gln-deprived Sp2/0 cultures treated with DON or AZA caused a marked increase in cell viability. More importantly, the combination of ammonium salts and Gln antagonists resulted in a significant reduction in caspase-3 activation in Gln-starved Sp2/0 cells, indicating that they interfered with the apoptotic process. Altogether, our data demonstrate that ammonium ions trigger a pro-survival cellular response in Gln-deprived Sp2/0 cells.

Keywords

Ammonia, apoptosis, caspase, glutamine, glutamine addiction, glutamine analog, glutaminolysis, hybridoma.

STATEMENT OF CO-AUTHORSHIP

The author was the primary author of this thesis and under his supervision, Catherine Zhou performed Western blots of BCL-2 proteins (Figure 3.17 A/B).

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DEDICATION

I dedicated this thesis, however imperfect, to my late father Chief Judge of Benghazi's Appeal Court, Mohammed Abusneina for planting the interest of knowledge and science in me; may Allah have mercy on you. To my wonderful mother, Samira Zedan, who despite the lands and seas that separate us, she always was here encouraging and supporting me, and also to my wife and my dear children, Muhammad, Albatoul, Maram and Kamal.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Symbol	Definition
AA	Ammonium acetate
AC	Ammonium chloride
AIF	Apoptosis-inducing factor
αKG	Alpha-ketoglutarate
AlaAT	Alanine aminotransferase
AMPK	Adenosine 5’-monophosphate-activated protein kinase
ANOVA	Analysis of variance
APAF-1	Apoptotic protease-activating factor 1
ASCT2	ASC amino acid transporters 2
ASK1	apoptosis signal-regulating kinase 1
ASNase	Asparaginase
AZA	O-diazoacetyl-L-serine (Azaserine)
BAD	Bcl-2 antagonist of cell death
BAK	Bcl-2 antagonist/killer
BAX	Bcl-2 -associated X
BCL-2	Bcl-2 lymphoma-2
BCL-W	Bcl-2 like protein 2
BCL-X_L	B-cell lymphoma-extra large
BH	Bcl-2 homology (domain)
BID	BH3 interacting-domain death agonist
BIK	Bcl-2 interacting killer
BIM	BCL-2-interacting mediator of cell death
BMF	Bcl-2-like modifying factor
BSA	Bovine serum albumin
CARD	Caspase-associated recruitment domain
CYT-C	Cytochrome c
DD	Death domain
DED	Death effector domain

DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	6-diazo-5-oxo-L-norleucine
DR	Death receptor
DRD3	Dopamine receptor D3
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENDO-G	Endonuclease G
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain protein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLIP	FLICE-inhibitory protein
GADD-153	Growth arrest and DNA damage-inducible gene 153
GDH	Glutamate dehydrogenase
GFAT	Glutamine-fructose-6-phosphate amidotransferase
GlcNAc	N-acetylglucosamine
Gln	L-glutamine
GLS	Glutaminase
GSH	Glutathione
HIF-1	Hypoxia-inducible factor-1
HO-1	Heme oxygenase-1
hPSCs	Human pluripotent stem cells
HSP60	Heat-shock protein 60
HSP70	Heat-shock protein 70
IAP	Inhibitor of apoptosis protein

IMDM	Iscove's-Modified Dulbecco's Medium
IMM	Inner mitochondrial membrane
IMS	Inner mitochondrial space
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LAT1	L-type amino acid transporter 1
LC3	Microtubular-associated light chain 3
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia 1
MeOH	Methanol
MOMP	Mitochondrial outer membrane permeability
MSO	Methionine sulfoximine
mTOR	Mammalian target of rapamycin
NAC	N-acetyl-L-cysteine
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NF_kB	Nuclear factor $_k$B
O/N	Overnight
OAA	Oxaloacetate
O-GLcNAc	O-linked β-N-acetyl-glucosamine
OMI/HtrA2	High-temperature requirement protein A2, serine protease, IAP antagonist
OMM	Outer mitochondrial membrane
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP ribose) polymerase
PBS	Phosphate buffered saline
PEP	Phosphoenolpyruvate

PFK	Phosphofructokinase
PGM	Phosphoglycerate mutase
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase.
PK	Pyruvate kinase
PKM2	Pyruvate kinase M2
PLA2	Phospholipase A2
PPP	Pentose phosphate pathway
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
PUMA	p53-upregulated mediator of apoptosis
RING	Really interesting new gene
RT	Room temperature
SA	Sodium acetate
SC	Sodium chloride
SCO2	Synthesis of cytochrome c oxidase 2
SDS	Sodium dodecyl sulfate
SIRT4	Sirtuin-4
SMAC	Second mitochondria-derived activator of caspases
Sp2/0	Sp2/0-Ag14
tBID	Truncated BID
TCA	Tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Transmembrane (domain)
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRADD	TNFR type 1-associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand

TTBS	Tris-buffered saline with Tween-20
UVB	Ultraviolet B
UDP-GlcNAc	Uridine diphosphate -N-acetylglucosamine
XIAP	X-linked inhibitor of apoptosis protein

Units of Measurement

°C	Degree Celcius
µg	Microgram
µl	Microlitre
µM	Micromolar
h	Hour
kDa	Kilodalton
M	Molar
min	Minute
mg	Milligram
ml	Millilitre
mM	Millimolar
SD	Standard deviation

1. INTRODUCTION

1.1 Metabolic adaptation in actively proliferating cells

One of the important factors for the cell's decision to continue proliferation or commit to apoptosis depends on the metabolic activity and availability of nutrients (1). Actively proliferating cells show increased bioenergetic and biosynthetic needs in order to duplicate cell organelles as well as replicate DNA (2). This process requires abundant amounts of molecular building blocks such as nucleotides, proteins and lipids. In order to satisfy this need, proliferating cells must reprogram their metabolism (3). Quiescent cells depend on mitochondrial oxidative phosphorylation to generate ATP via the tricarboxylic acid cycle (TCA) and only under low oxygen, convert glucose to lactate through glycolysis for ATP production. Proliferating cells, however, catabolize glucose mainly through glycolysis, even in the presence of sufficient oxygen. This phenomenon is known as the "Warburg effect" (Figure 1-1) (4-7). As a result, glucose is metabolized to lactate with only two molecules of ATP per glucose molecule being produced compared to the 36 ATP molecules obtained via oxidative phosphorylation. This shift of metabolic reprogramming was puzzling as proliferating cells require a large amount of energy to grow and divide and yet selectively rely on glycolysis as a path for ATP production (8). In 1927, Warburg reported that the cellular metabolic change observed in cancer cells was due to defective mitochondria, which in turn impaired the ability of these cells to utilize glucose efficiently. However, due to our recent understanding of cancer metabolism, we know now that, in most cancer types and in normal actively proliferating cells, mitochondria are not defective in performing oxidative

phosphorylation. Nevertheless, proliferating cells reprogram their mitochondrial function through anabolic metabolism in order to ensure rapid ATP production, and to generate a continuous supply of biosynthetic metabolites needed for rapid growth and proliferation (2,3,9).

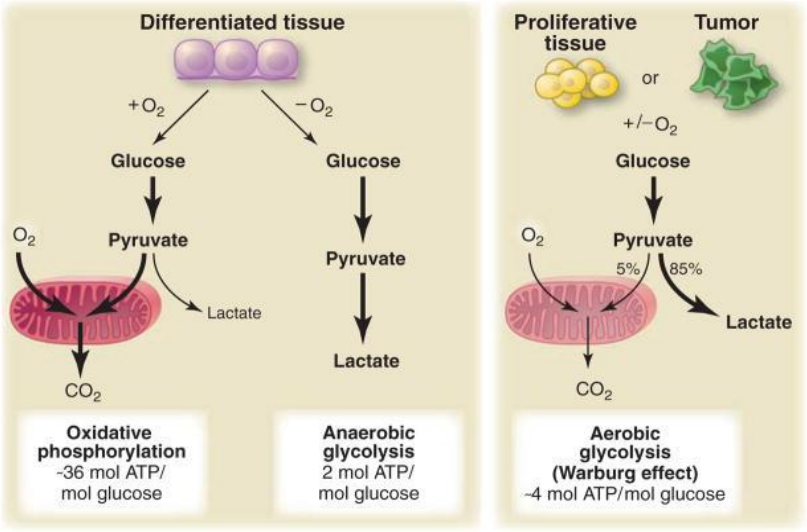


Figure 1-1 Warburg effect

In quiescent cells (left panel), glucose is metabolized through glycolysis and the tricarboxylic acid (TCA) cycle, generating 36 mol of ATP per mol of glucose. In contrast, in actively proliferating cells (right panel), glucose bypasses the TCA cycle in favor of biosynthesis to make membranes, amino acids and other macromolecules. From (9) Reprinted with permission from AAAS.

This change in cellular metabolism characterizing actively proliferating cells is an outcome of the modulation of signaling pathways of modulating energy metabolism, high glucose uptake, as well as mitochondrial activities (10,11). Actively proliferating cells achieve this by overexpressing cell membrane glucose transporters (Glut-1) which allow increased glucose uptake. For instance, frequent activation of the Phosphoinositol 3-kinase/protein kinase B (PI3K/AKT) pathway in cancer leads to increased

translocation of glucose transporters from intracellular vesicles to the cell membrane (12). Another way actively proliferating cells reprogram their metabolism is by increasing the expression of glycolytic enzymes and/or their isoforms, such as hexokinase (HK), pyruvate kinase (PK), phosphofructokinase (PFK) and others (13). PK, in particular, is very important as it catalyzes the reaction that converts phosphoenolpyruvate (PEP) to pyruvate, a step in glycolysis that produces ATP. There are different isoforms of PK, but the PKM2 isoform is predominantly expressed in cancer and proliferating cells (14) and is less effective at catalyzing the conversion of PEP to pyruvate. This results in accumulation of PEP, making upstream metabolites available to be shuffled into the pentose phosphate pathway (PPP) for the production of anabolic precursors, e.g., ribose and NADPH (13,14). The latter is needed for biosynthesis of macromolecules, such as fatty acids and cholesterol (9,15).

This metabolic reprogramming is also modulated by (proto) oncogenes and tumor suppressors (Figure 1-2). In particular, changes in signaling pathways involving PI3K/AKT, p53 and Phosphatase and tensin homolog (PTEN) have been shown to be key to metabolic reprogramming (16-18). PI3K is a protein kinase whose activity is initiated by the activation of receptor tyrosine kinases. It leads to the activation of Akt, a serine-threonine protein kinase that activates several downstream targets such as FOXO and mTOR which dictate cell survival and proliferation responses (19-21). PI3K signaling also modulates mTORC1, a protein kinase complex which is implicated in

regulating many aspects of metabolic pathways as well as the promotion of protein synthesis and growth (22,23).

Tumor suppressors also participate in regulating the metabolic adaptation observed in actively proliferating cells. For example, p53 inactivation can increase the levels of phosphoglycerate mutase (PGM) a glycolytic enzyme required for conversion of 3-phosphoglycerate to 2-phosphoglycerate. This leads to elevated levels of glycolysis (24,25). Also, p53 can stimulate oxidative phosphorylation by inducing cytochrome c oxidase 2 (SCO2) synthesis and decrease the production level of reactive oxygen species (ROS) in the cell (26,27). Finally, PTEN, by acting as an inhibitor of PI3/AKT pathway, regulates the metabolic shift from oxidative phosphorylation to glycolysis (28).

1.2 Glutaminolysis and its importance in the metabolism of actively proliferating cells

As a consequence of the metabolic adaptations described above, glucose contributes less carbons to the TCA cycle. To replenish the latter, actively proliferating cells shuffle glutamine to the mitochondria where it is oxidized first to glutamate (via the enzyme glutaminase – GLS) and then to α-ketoglutarate (αKG) via glutamate dehydrogenase (GDH), a process known as glutaminolysis (Figure 1-3) (29,30). αKG can then be used to fuel the TCA cycle.

In addition to its role in replenishing the TCA cycle, glutamine is used as a precursor for the synthesis of several molecules essential to actively proliferating cells, such as NADPH, ribose, lipids and amino acids (Figures. 1.2 and 1.3) (31). This requirement for glutamine by actively proliferating has been termed “glutamine addiction” (32).

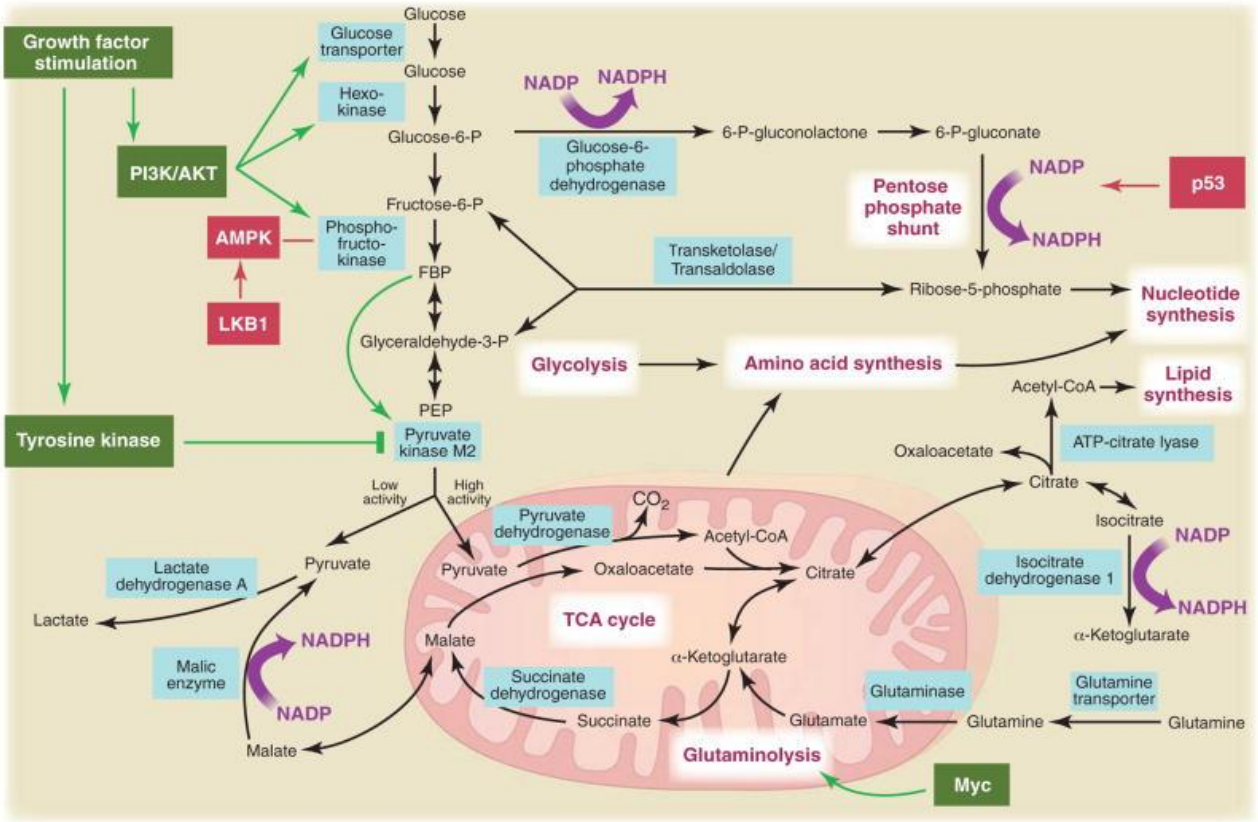


Figure 1-2 Reprograming of metabolism in actively proliferating cells.

A schematic representation shows the interaction between the oncogenes and tumor suppressors and signaling pathways that modulate different metabolic pathways in proliferating cells. From (9) Reprinted with permission from AAAS.

1.2.1 The modulation of glutaminolysis in actively proliferating cells

In actively proliferating cells, glutaminolysis is tightly modulated by several factors, including p53, K-RAS and, most notably, MYC (16,33,34).

1.2.1.1 p53

p53 stimulates glutaminolysis by increasing Glutaminase 2 (GLS2) levels (33,35). Interestingly, in contrast to GLS1, which is associated with increased proliferation, GLS2 is tumor suppressive (33,35). The molecular mechanism underlying this difference is unknown but may be linked to functions of these proteins unrelated to glutamine metabolism: for example, GLS2 has recently been shown to participate in the modulation of gene expression (36,37).

1.2.1.2 K-RAS

This oncogene has been shown to increase glutaminolysis through two different routes. Firstly, by increasing the expression of GDH, K-RAS ensures a sufficient input of glutamine-derived carbons into TCA cycle. Along with other changes in gene expression leading to the use of glycolytic intermediates in anabolic pathways (38), K-RAS expression results in a decoupling of glucose and glutamine metabolism in actively proliferating cells, allowing the latter to satisfy their requirements in biosynthetic precursors (39).

Secondly, K-RAS triggers the up-regulation of aspartate transaminase and, as a result, promotes the conversion of aspartate to oxaloacetate. The latter is then transformed into malate, which is used by malic enzyme to produce pyruvate and, importantly, NADPH (40).

1.2.1.3 MYC

As a transcription factor, MYC is known to modulate the expression of several genes required for the cell cycle and its regulation (e.g. Cyclins A, D1 and E; CDC25; $p15^{INK4B}$), DNA synthesis (thymidine kinase) as well as metabolism (LDH-A) (41,42). More recently, MYC expression was shown to trigger the expression of genes responsible for increasing glutamine metabolism in proliferating cells (43,44). The role of MYC in modulating metabolic reprogramming was elegantly demonstrated by Wang et al. (43). Using mouse primary T cells, this group showed that T cell activation (by treatment with an anti-CD3 antibody) triggered a metabolic reprogramming typical of actively proliferating cells (i.e. aerobic glycolysis, increased glutaminolysis, increased glucose metabolism through the pentose phosphate pathway). Importantly, in T cells in which the MYC gene was deleted using a cre-lox system, both the metabolic reprogramming and cell proliferation upon activation were compromised (43).

The control of glutamine metabolism by MYC in actively proliferating cells occurs at multiple levels. MYC increases the expression of the glutamine importers SLC1A5, SCL38A5 in a mechanism that apparently involves increased transcription (44). Moreover, MYC downregulates the expression of the miR-23a microRNA, which is itself

a negative regulator of GLS-1, and the glutamine importer SLC6A14 (45,46). MYC has also been shown to trigger the expression of glutamine synthetase (47), GLS-2 (48) and GDH (43). It is therefore unsurprising that increased expression of MYC is a major contributor to glutamine addiction in proliferating cells.

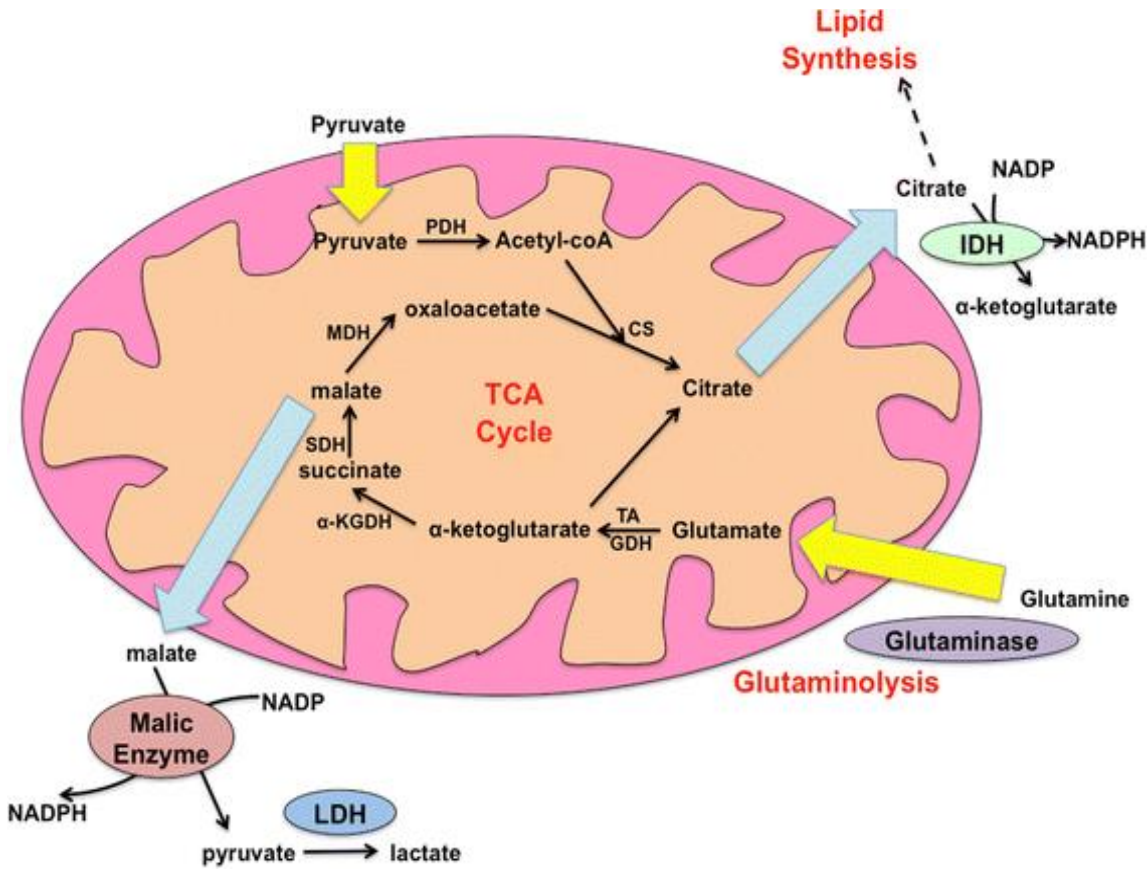


Figure 1-3 Glutaminolysis in actively proliferating cells

In cancer and actively proliferating cells, glutamine is transported into the cell and is metabolized by glutaminase (GLS) to glutamate and ammonia. Glutamate is then converted to α-ketoglutarate by glutamate dehydrogenase (GDH) and enters the TCA cycle where it is metabolized to oxaloacetate (OAA). The latter condenses with acetyl-CoA to form citrate. Malate produced during glutamine metabolism is converted to pyruvate by malic enzyme, generating NADPH. Pyruvate is subsequently metabolized to lactate by LDH resulting in NADH production (49). Reprinted under Creative Commons Attribution 4.0 International License.

1.2.2 Relevance of glutamine metabolism to cancer therapy

Because of the central role played by glutamine in the metabolism and intracellular signaling of proliferating cells, the possibility of targeting glutamine metabolism as a way to cancer therapy has been suggested (Figure 1-5) (31,32,50,51). Therapeutic approaches can include depletion of glutamine from the blood, blocking glutamine transportation across the cell membrane, use of glutamine analogs or decrease the activity of enzymes necessary for glutamine metabolism (31).

For example, the bioavailability of glutamine can be lowered by the use of phenylbutyrate which has been shown to prevent cancer cells from proliferating (52). In humans, phenylbutyrate is metabolized to phenylacetate, which in turn binds to glutamine to form phenylacetylglutamine which is excreted from the body (52-55). Use of L-asparaginase (ASNase), an enzyme that catalyzes the removal of the amide nitrogen from glutamine to form glutamic acid, is a part of the standard chemotherapeutic program to treat pediatric acute lymphoblastic leukemia (ALL), has proven to deplete glutamine by approximately 90% in the blood of ALL patients (56,57). Targeting the glutamine transporter, SLC1A5 by the L-glutamine analog l-γ-glutamyl-p-nitroanilide, has been shown to inhibit cell growth and viability in lung cancer and human colon carcinoma cells (54,58,59).

1.2.2.1 Glutamine analogs

The dependence of certain cancer cells toward glutamine has incited scientists to test structural analogs of this amino acid as a possible chemotherapy agent (51). Glutamine analogs act as irreversible inhibitors of glutamine-dependent enzymes by covalently attaching to the catalytic centers of these enzymes (51). Glutamine analogs were used to target glutamine in cancer therapy. In clinical trials, it was revealed that 6-diazo-5-oxo-l-norleucine (L-DON) and azaserine (AZA), two glutamine analogs (Figure 1-4), were able to cause cytotoxicity *in vivo* in certain tumors. However, due to their severe toxicity, their use was discontinued (32,54,60,61). Nevertheless, since both DON and AZA block glutamine catabolism, they are frequently used to study the molecular basis of glutamine addiction (62).

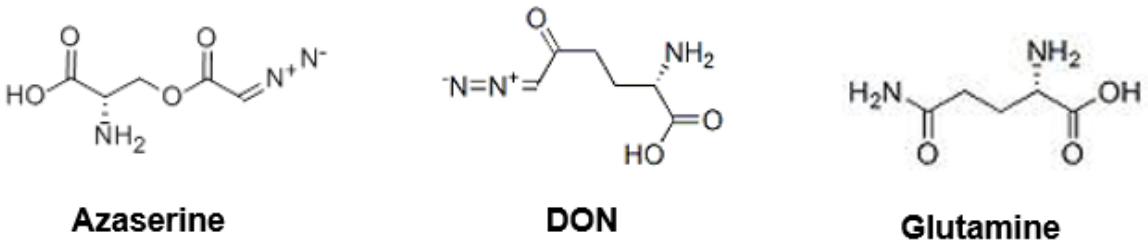


Figure 1-4 Structure of azaserine and DON

1.2.2.1.1 6-Diazo-5-oxo-L-norleucine (DON)

DON was firstly described in 1956 as an antibiotic with antitumor activity isolated from an unidentified soil bacterial *Streptomyces* strain (63,64). DON has been used as an inhibitor of enzymes that utilize glutamine such as CTP synthetase (CTPS), carbamoyl phosphate synthase (CAD), glutaminase and others. These enzymes are involved in metabolic pathways such as the synthesis of purine, pyrimidine and amino acids (60,63,65-68). The effect of the antitumor capability of DON was investigated in several studies (68). DON was found to be effective in reducing several tumors if used alone for example in murine leukemia or with combination with azaserine in the case of mammary murine carcinoma (69) Recently, it was reported that the combination of DON with polyethylene glycol-conjugated glutaminase (PEG-PGA), a glutamine depleting enzyme, was very effective in a phase-II clinical study on advanced refractory tumors (70). DON can perturb the cell cycle as it causes S-phase arrest in some normal and neoplastic human cell lines (71). DON can also interfere with the function of cell organelles. DON was reported to cause disruption of the mitochondrial inner membrane in a neuroblastoma cell line as well as swelling of the endoplasmic reticulum, nuclear condensation, DNA strand cleavage and even apoptosis (72,73). DON can also inhibit growth in human carcinoid tumors and induce apoptosis through BAX in neuroblastomas and Ewing’s sarcoma cell lines expressing high levels of MYC (74,75).

1.2.2.1.2 O-diazoacetyl-L-serine (Azaserine)

Azaserine is the most studied glutamine analog. Azaserine like DON, has antitumor activity and interferes with several metabolic processes within the cell. Azaserine irreversibly inhibits glutamine amidotransferase, an enzyme required for ATP-dependent removal of the amido group from glutamine and transfers it to a specific acceptor. Azaserine also inhibits purine and cytidine synthesis (76,77). Azaserine was also reported to inhibit L-glutamine-D-fructose 6-phosphate amidotransferase (GFAT), an enzyme which catalyzes the conversion of fructose-6- phosphate to glucosamine-6-phosphate using glutamine as the amine donor. Glucosamine-6-phosphate is then metabolized through the hexosamine pathway, leading to the formation of UDP-GlcNAc, a precursor for the synthesis of glycoproteins, glycolipids and proteoglycans (78,79).

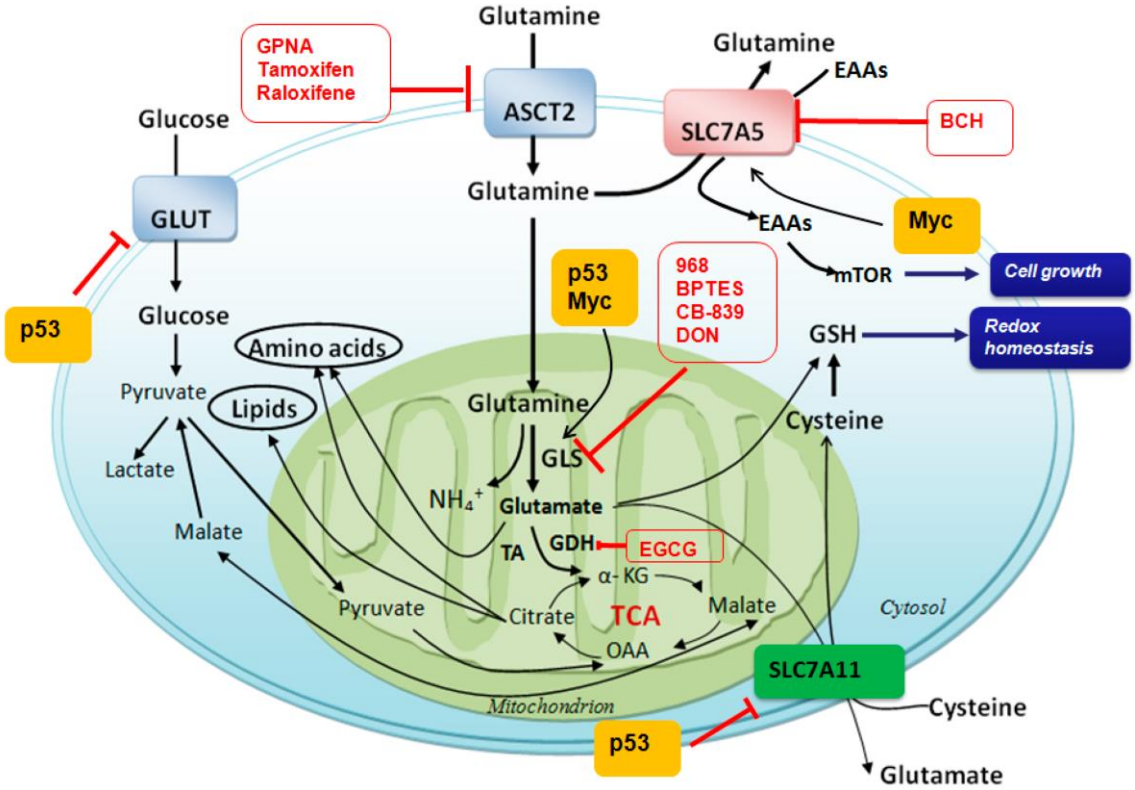


Figure 1-5 Glutamine metabolism and possible cancer therapeutic targets

Glutamine is transported into the cell and is metabolized to glutamate and ammonium ions by glutaminase which can be used for the production of building blocks required by the cell such as lipids and amino acids which in turn lead to cell growth via activation of the mTOR pathway. Several steps and components of glutamine metabolism can be targeted for cancer therapy which can be blocked by several pharmacological inhibitors. LAT1: L-type amino acid transporters 1, ASCT2: system ASC amino acid transporters 2, GLS: glutaminase, GDH: glutamate dehydrogenase. Reprinted under the Creative Commons Attribution License (31).

1.2.3 Glutamine and apoptosis

Both normal and cancerous actively proliferating cells have been shown to depend on glutamine not only for growth and proliferation but also for their survival. In both rat and human neutrophils, glutamine has been shown to impede the initiation of apoptosis (80). Glutamine withdrawal triggers apoptosis in rat intestinal epithelial cells (81). Moreover, human pluripotent stem cells (iPSC) are dependent on glutamine metabolism for their survival (82). Several cancer cell lines (HEY, SKOV3, SF-188, MDA-MB-231 and SUM149) have been shown to undergo apoptosis upon glutamine deprivation (44,83,84).

In order to better understand the role which glutamine plays on the control of apoptosis, a brief review of this mode of cell death is described below. I then describe some of the proposed mechanisms responsible for the modulation of cell survival by glutamine.

1.2.3.1 Apoptosis

Apoptosis, a type of programmed cell death (PCD), has many roles in multicellular organisms. Apoptosis controls cell number during organism development and is a homeostatic mechanism by which cell population is maintained. Apoptosis is also the mean by which the immune system can rid the body of diseased or infected cells (1,85). Apoptosis is a process in which enzymes belonging to a family of cysteine-aspartic proteases (caspases) are activated either from within the cell or externally which leads

to distinctive morphological changes. These changes include nuclear fragmentation, cell shrinkage, chromatin condensation, membrane blebbing and the formation of apoptotic bodies. The latter are subsequently engulfed and digested by macrophages (86-88). The distinctive morphological features of apoptosis are the result of biochemical reactions that happen in the cell. The main biochemical characteristics of apoptosis are:

1) Externalization of phosphatidylserine (PS) from the inner side of the cell membrane bilayer. This externalization of PS allows the phagocytotic cell to systematically recognize apoptotic cells to eliminate them from the body (89). This event can be specifically detected by staining the cells with a fluorescently-labeled Annexin-V protein, which binds to PS. Annexin-V cannot enter through the intact cell membrane. Therefore, fluorescence detection can be used to detect the presence of apoptotic cells (for example, by flow cytometry or fluorescence microscopy) (90).

2) Activation of initiator caspases which leads to subsequent activation of other, executioner caspases. The latter mediate the proteolytic cleavage of their intracellular substrates. Each caspase is activated in a specific step during apoptosis, and each caspase has several specific intracellular substrates (91). The caspase cleavage of substrates can be detected by different methods such as Western blot or the colorimetric detection of the cleavage of synthetic caspase substrates (1,92).

3) During apoptosis, the genomic DNA is cleaved into fragments containing units of

approximately 140 nucleotides. This fragmentation can be seen as a ladder bands of fragmented DNA when using agarose gel electrophoresis (88).

Apoptosis induction in mammals occurs through two main signaling pathways (93). The intrinsic or mitochondrial pathway, which can be triggered by different conditions within the cell including oxidative stress or DNA damage, converges at the mitochondria and leads to the formation of the apoptosome complex, which is responsible for the activation of the initiator caspase 9 (1). In contrast, the extrinsic pathway is initiated by stimuli from outside the cell which acts on the cell surface death receptors, leading to the activation of downstream initiator caspases 8 or 10 (Figure 1-6) (94).

1.2.3.1.1 Intrinsic apoptotic pathway

The intrinsic pathway can be triggered by numerous intracellular stimuli including DNA damage, cytoskeleton disruption, hypoxia and withdrawal of growth factors or nutrients (94). Responding to any of these apoptotic stimuli, pro-apoptotic BCL-2 family members are activated and lead to a critical step in the intrinsic pathway, the mitochondrial outer membrane permeabilization (MOMP). The initiation of outer membrane permeabilization is mainly mediated by the BCL-2 family protein members BAX and BAK, which oligomerize to form pores at the mitochondrial outer membrane. A model called “rheostat” was suggested where the balance between anti-apoptotic and pro-apoptotic BCL-2 proteins act to regulate the involvement of mitochondria in apoptosis (95-98).

Once activated, BAX inserts itself into the outer mitochondrial membrane and oligomerizes with BAK to form pores, leading to MOMP (99). MOMP allows the release of a number of pro-apoptotic proteins from the mitochondrial intermembrane space (IMS) into the cytosol where they exert their role in the apoptotic cascade. Among these proteins are cytochrome c, Second Mitochondrial-Derived Activator of Caspases (SMAC/DIABLO), Apoptosis Inducing Factor (AIF), High-Temperature-Requirement Protein A2 (HTRA2/OMI) and Endonuclease G (Endo-G) (100,101).

Once it is released into the cytosol, cytochrome c binds to apoptotic protease activation factor-1 (APAF-1). APAF-1 is an adaptor protein that contains a caspase-associated recruitment domain (CARD) and is present in the cytosol in an inactive form (102). The binding of cytochrome c to APAF-1 leads to a conformational change in APAF-1 exposing the CARD domain which consequently promotes the CARD-CARD interaction with pro-caspase-9 to form a structure called the “apoptosome” resulting in caspase-9 activation (103-105). Now, the active initiator caspase-9 is able to activate effector caspases, e.g., caspase-3 and caspase-7, which in turn cleave various cellular substrate leading to progression of apoptosis and ultimately cell death (106). Caspase-3 can also activate other caspases including caspase-2, -6, -8 and -10 thereby propagating the apoptotic signal (107). X-linked inhibitor of apoptosis protein (XIAP) proteins can interfere with this process and inhibit the activity of caspase-3, -7 and -9 (108).

1.2.3.1.2 Extrinsic pathway

Activation of the extrinsic apoptotic pathway (Figure 1-6) is mediated by cell transmembrane receptors, known as Death Receptors (DR) which, upon binding to specific ligands, transmit an apoptotic signal inside the cell (1). Death receptors belong to the tumor necrosis factor receptor (TNFR) family and include TNF-R1, TRAIL, and FAS, among others (109). They contain a cysteine-rich extracellular domain, necessary for specific ligand binding, and a cytosolic 80 amino acid domain called Death Domain (DD) (110,111). This family also includes the ligands of these receptors namely FASL, TNF and TRAIL.

After ligand binding, death receptors trimerize and undergo a conformational change exposing their DD. The DD recruits a DD- containing adapter protein: while FASL/FAS binding recruits FAS-associated death domain protein (FADD), TNF/TNF-R recruits TNF-R1-associated death domain protein (TRADD). Then, FADD and TRADD, associated with pro-caspase-8 through a death effector domain (DED), leads to the formation of the death-inducing signaling complex (DISC) (112,113). In this complex, through close proximity, procaspase-8 is autocatalytically cleaved and activated (112,114,115). When activated, caspase-8 directly cleaves and activates caspase-3 (116) resulting in the induction of the caspase cascade in the cell. Activation of caspase-8 can also involve the intrinsic pathway via cleavage of BID to a truncated form (tBID). tBID then translocates to the mitochondrial outer membrane (OMM) and induces MOMP, allowing the efflux of the pro-apoptotic proteins to the cytosol (115,117). However, activation of caspase-8 via DISC complex can be inhibited by the cellular

FLICE-like inhibitory protein (cFLIP) which also contains a DED domain by which it can block the recruitment of caspase-8 by the adaptor protein thereby inhibiting caspase-8 activation (118).

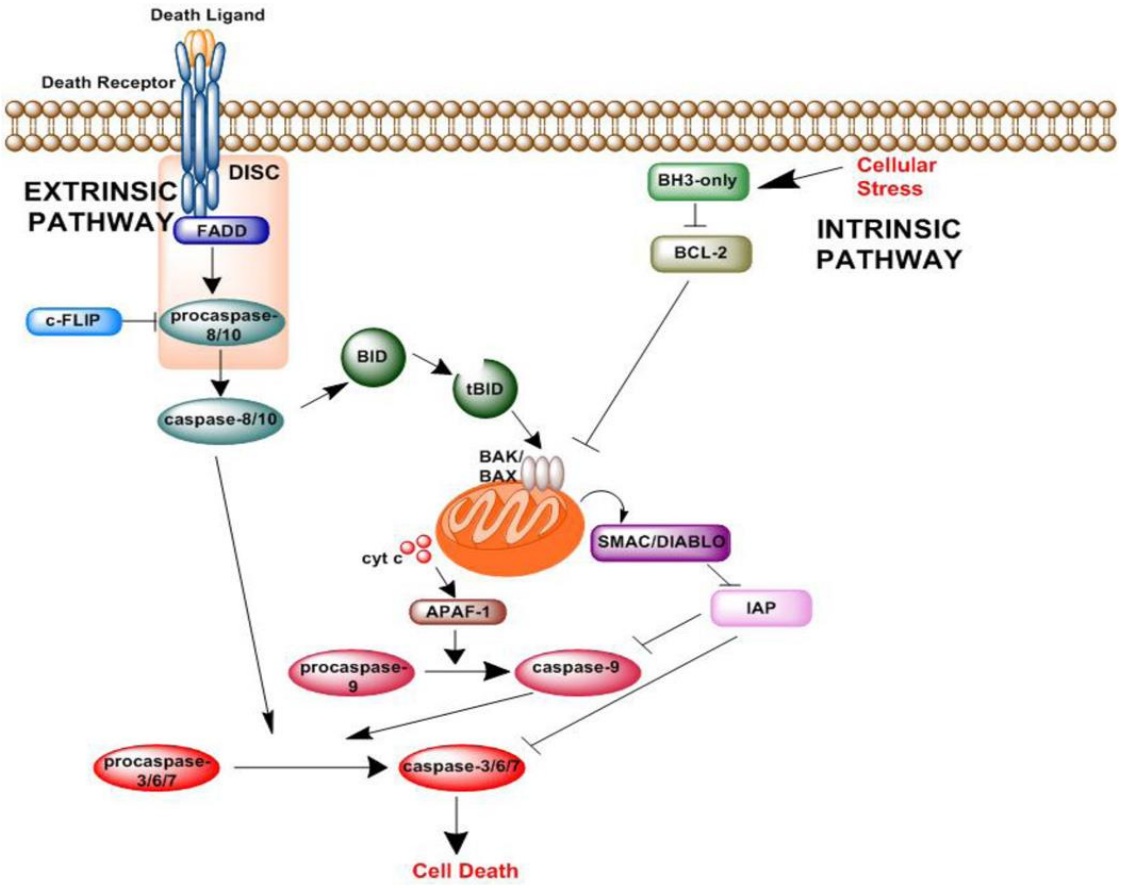


Figure 1-6 The extrinsic and intrinsic apoptotic pathways

Apoptosis can be triggered upon binding of death ligand to death receptor which initiates extrinsic pathway and activation of death-inducing signaling complex (DISC). Caspase-8 can directly activate downstream caspases or cleave Bid. The intrinsic pathway is modulated by mitochondria and Bcl-2 proteins in response to various physiological signals. Upon the activation of pro-apoptotic Bcl-2 proteins, outer mitochondrial membrane permeabilization occurs and proapoptotic proteins, such as cytochrome c are released. Cytosolic cytochrome c binds apoptotic protease activating factor 1 (Apaf-1 and recruits procaspase-9 to form the apoptosome. Caspase-9 activates downstream caspases (119).

1.2.3.2 Role of glutamine in apoptosis

1.2.3.2.1 Modulation of the extrinsic pathway.

Glutamine deprivation has been demonstrated in some cell lines to cause the induction of the extrinsic apoptotic pathways. In human monocytic U937 cells, glutamine deprivation significantly increased the cellular sensitivity to TNFα and FASL (120). In the hybridoma cell line CRL-1606, glutamine starvation causes the up-regulation of FAS, along with the activation of caspase-8 (121).

In CEM and HL-60 cells, glutamine deprivation triggers apoptosis through the FAS-mediated activation of the extrinsic pathway (122). This process occurred in ligand-independent manner. Because supplementation of glutamine-deprived cells with D-glutamine could prevent the induction of apoptosis, the authors concluded that glutamine deprivation caused an osmotic stress. The resulting cell shrinking led to the clustering of FAS molecules and the subsequent activation of caspase-8.

In another study, Ko et al. (123) have shown that glutamine deprivation sensitized HeLa cells to FASL-induced apoptosis. Interestingly, the underlying mechanism involved glutaminyl-tRNA synthetase (QRS), the enzyme responsible for charging the $tRNA^{Gln}$ with glutamine. What Ko et al. uncovered is that in the presence of glutamine, QRS associated with and inhibited ASK1, a MAPKK activated as part of FAS-mediated apoptosis. Interestingly, glutamine deprivation disrupted the ability of QRS to inhibit the

activation of ASK1, providing an explanation for the increased cellular sensitivity to FASL.

1.2.3.2.2 Modulation of the intrinsic pathway

The observation that supplementation with glucose, but not glutamine, protects human neutrophils against FAS-induced apoptosis indicates that the modulation of the latter by glutamine is cell type-dependent, and that other cell death pathways are subjected to regulation by this amino acid (124). Effectively, a number of studies have revealed the induction of the intrinsic pathway following glutamine withdrawal (125).

Using human and rat neutrophils, Pithon-Curi et al. observed that the induction of apoptosis following glutamine deprivation was associated with a decrease in mitochondrial transmembrane potential, indicating a loss of mitochondrial function (80). In human Jurkat T cells, glutamine supplementation led to an increase in BCL-2 expression and in cell viability (126). Likewise, in several hybridoma cell cultures, glutamine deprivation triggers apoptosis that is inhibited by the ectopic expression of BCL-2 family members (127,128). Finally, glutamine starvation-induced cell death was shown to require BAX/BAK (129,130), to lead to caspase-9 activity (121,131,132) and to decrease the expression of HSP70, a heat shock protein known to impede intrinsic apoptosis at multiple levels (133-135).

1.2.3.3 Possible mechanisms of apoptosis induction by glutamine deprivation

Aside from a few examples mentioned above where the experimental evidence suggests a possible mechanism (122,123), the molecular processes whereby glutamine promotes cell survival remain poorly understood. In particular, it is unclear how the cell senses glutamine levels and relays that information to the intrinsic apoptotic machinery to inhibit cell death. In the following section, I describe a number of possible ways whereby glutamine has been shown to modulate cell viability (Figure 1-7).

1.2.3.3.1 Metabolism

Considering its importance as a major energy source of actively proliferating cells, one would expect the modulation of survival by glutamine to be dependent on its catabolism.

Using CEM human leukemia cells, Petronini et al. demonstrated that, while glutamine starvation triggered apoptosis, the extent of cell death was not affected when the glutamine-free medium was renewed daily (136). This suggested that glutamine deprivation did not trigger cell death through a decrease in intracellular energy levels. Confirming this study, Yuneva et al. showed that, while the combination of 2-deoxyglucose (an inhibitor of glycolysis) and antimycin A (a potent inhibitor of oxidative phosphorylation) led to a much greater decrease in ATP levels in human intestinal epithelial cells than glutamine starvation, the latter was much more potent at inducing apoptosis (132).

Recently, Zhang et al. provided evidence that the knock down of citrate synthase prevented the induction of apoptosis in glutamine-deprived SF188 human glioblastoma cells (130). This indicated that the pro-survival function of glutamine did not depend on its oxidation through the TCA cycle. On the other hand, these investigators showed that cell viability in the *presence* of glutamine was compromised when the expression of the enzyme asparagine synthase (which synthesizes asparagine from aspartate and glutamine) was decreased by RNAi. Moreover, the induction of apoptosis following glutamine deprivation could be prevented by the simple supplementation of asparagine to the culture. How asparagine controls cell death has not yet been determined in detail, but Zhang et al. identified GADD153 as a pro-apoptotic protein the expression of which is downregulated by asparagine (130). Thus, at least in SF188 cells, glutamine modulates cell survival by ensuring an adequate supply of asparagine.

1.2.3.3.2 Oxidative stress

As a biosynthetic precursor of glutathione (GSH), glutamine plays a key role in protecting cells against oxidative stress (137). In a number of cell lines, including the HuH-7 hepatoma and Jurkat T cells, glutamine promotes cell viability through an increase GSH levels (126,138). However, this effect is cell-type dependent. For instance, work from our laboratory with the mouse hybridoma Sp2/0-Ag14 (Sp2//0) has revealed that, while glutamine deprivation triggered an oxidative stress that can be mitigated by the antioxidant N-acetyl-L cysteine (NAC), NAC treatment did not result in an increase in cell survival. Moreover, reducing GSH levels with the GSH synthesis inhibitor buthionine-[S,R]-sulfoximine (BSO) did not trigger Sp2/0 cell death (139), an

observation also made by Yuneva et al. in glutamine-deprived human intestinal epithelial cells (132). Interestingly, NAC could prevent the formation of apoptotic bodies and nuclear condensation and fragmentation in glutamine-deprived Sp2/0 cells, a phenomenon that involved the stress-activated protein kinase p38 (140). This suggests that, while it is not essential to the loss of cell viability, oxidative stress triggered by glutamine starvation may still play a role in modulating some of the late morphological events leading to apoptosis (139).

1.2.3.3.3 The integrated stress response

When challenged with certain stresses, the cell triggers an Integrated Stress Response (141,142). This phenomenon involves the activation of an eIF2α-targeting, stress-specific protein kinase: PERK (endoplasmic reticulum stress), PKR (viral infection), (HRI) (heme deprivation), and GCN2 (amino acid starvation) (141,142). Upon activation, these kinases will trigger a sequence of signaling events which will allow the cell to elaborate a stress response.

Following amino acid starvation, GCN2 is activated in response to the accumulation of uncharged tRNAs (143,144). Upon activation, the ribosome-bound GCN2 phosphorylates the alpha subunit of the translation initiation factor eIF2, locking the latter into a GDP-bound state and preventing cap-dependent translation initiation. Concurrently, a number of mRNAs containing short upstream open reading frame (uORF) in their 5' untranslated region (5' UTR) are translated. One of these mRNAs encodes the transcription factor ATF-4, which acts as a homodimer or as an

heterodimer with other protein partners (including one of its gene targets, CHOP-10/GADD153) to modulate new gene transcription and lead to a stress adaptation response (141,142). A milder stress causes the up-regulation of amino acid transporters and biosynthetic enzymes, thus allowing the cell to survive. On the other hand, a more severe stress increases the expression of apoptosis-related proteins and results in cell death (141,142,145).

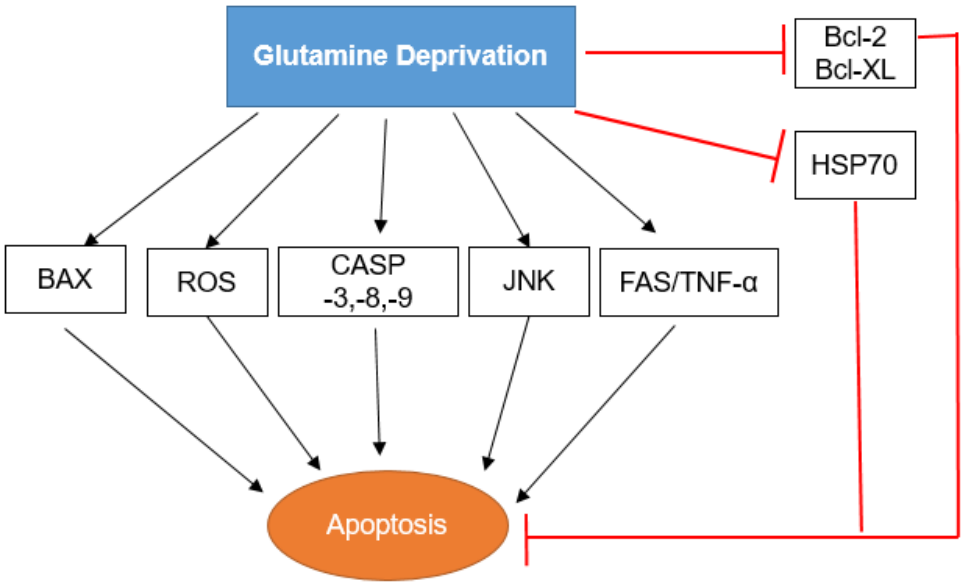


Figure 1-7 Role of glutamine deprivation in apoptosis

Apoptosis can be triggered by glutamine deprivation via different pathways. Glutamine deprivation can sensitize cells depending on cell type to the intrinsic pathway through mitochondria leading to activation of caspase-3 or extrinsic apoptosis pathway through Fas and TNF-α ligands that leads to activation of caspase-8. Glutamine deprivation sensitizes cells to JNK, ROS as well as inhibits HSP70. ROS: reactive oxygen species, JNK: c-Jun N-terminal kinase, HSP70: heat shock protein 70. Red ⊥: inhibiting effect.

Several studies have linked the cellular response to glutamine starvation with the Integrated Stress Response. Acute glutamine deprivation has been shown to trigger GCN2 activation and/or eIF2α phosphorylation in several human cancer cell lines (129,146,147). Moreover, glutamine was shown by several teams to modulate the expression of CHOP-10 (130,147-149). Finally, Qing et al. demonstrated that the induction of apoptosis in glutamine-starved MYC-overexpressing cells was dependent on ATF4 and the ability of the latter to up-regulate the expression of the BH3-only proteins PUMA and NOXA (129).

1.2.3.3.4 Glutamine and mTOR

The mechanistic target of rapamycin (mTOR) protein kinase is at the core of a signaling pathway which responds to a variety of signals (e.g. low ATP levels, DNA damage, growth factors, hypoxia, nutrients) and uses this information to modulate cell proliferation, metabolism, autophagy and cell survival (150). For the sake of brevity, I have restricted my discussion on how the mTOR pathway responds to glutamine levels and how this can lead to a survival response.

In the cell, mTOR forms two structurally and functionally distinct complexes, mTORC1 and mTORC2, with mTORC1 being the best characterized of the two. In addition to mTOR, mTORC1 includes two other subunits, Raptor and mLST8. mTORC1 is recruited to the lysosome and requires its binding to RAG proteins, which are associated with the lysosomal membrane. mTORC1 is then activated by its amino acid-

dependent association with Rheb, a guanine nucleotide exchange factor (GEF) protein localized to the lysosomes (145,150). Upon activation, mTORC1 phosphorylates a number of protein targets, leading to protein, lipid and nucleotide synthesis and to the inhibition of autophagy.

mTORC2 is composed of three subunits: mTOR, Rictor and mLST8, and promotes cell proliferation and survival (150,151). The known protein substrates of mTORC2 are limited, and include AKT, SGK and members of the PKC family. In contrast to mTORC1, mTORC2 is insensitive to acute treatment by the antibiotic rapamycin, a property which is often used to distinguish between the contribution of these two protein complexes to specific cellular processes.

Recent work has revealed that glutamine modulates the function of both mTORC1, with consequence to cell survival. The cellular efflux of glutamine through the LAT1 antiporter causes the influx of the amino acid leucine, a well-known mTORC1 pathway stimulator (152,153). Moreover, αKG, produced from glutaminolysis, has been shown to be an activator of mTORC1 (152). Glutamine also promotes the recruitment of mTOR to the lysosome in a RAG-independent manner, via still undefined mechanisms involving the RAS family member ADP-ribosylation factor 1 (ARF1). Glutamine deprivation has been shown to lead to mTOR inhibition and to stimulate autophagy, a process through which cells digest their own organelles in order to obtain nutrients (154).

1.3 Research rationale, hypothesis and objectives

The research program in our laboratory focuses on the elucidation of the cellular and molecular events underlying the modulation of cell survival by glutamine. I will first describe the cell model used in our studies. I will then provide the rationale to my research project, propose a working hypothesis and describe my research objectives.

1.3.1 The Sp2/0-Ag14 cell line as a model to study the effect of glutamine on cell survival

To investigate the modulation of cell survival by glutamine, our research group has used the mouse hybridoma cell line Sp2/0. This cell line has two interesting properties that make it a useful model to study the modulation of apoptosis by glutamine (131):

1) It rapidly undergoes apoptosis upon glutamine starvation, with typical apoptotic morphological features being observed only 2 hours after the removal of the amino acid;

2) Rapid Sp2/0 cell death is not observed when the cells are deprived of any of the other 19 amino acids.

Efforts by our research group have revealed that glutamine starvation rapidly activates the intrinsic pathway in Sp2/0 cells: the release of the mitochondrial apoptogenic proteins CYT-C and SMAC/DIABLO is observed within 30 mins of glutamine withdrawal, followed by the recruitment of Bax to mitochondria and the activation of caspase-9 and

caspase-3 (131). Moreover, the glutamine-induced Sp2/0 cell death is potently inhibited by pro-survival BCL-2 family proteins (155).

The acute response of Sp2/0 cells to glutamine starvation has been attributed to a combination of three features of this cell line:

1) *The low expression of GS*: The Sp2/0 cells line, as well as hybridomas derived from it, have low expression of GLS (156). This limits the ability of these cells to synthesize glutamine, rendering them particularly vulnerable to the effects of glutamine starvation.

2) *Deregulated MYC expression*: In several mouse hybridomas, including the Sp2/0 cell line, MYC is expressed in a constitutive manner (128). In addition to its role in promoting metabolic reprogramming and glutamine addiction, MYC is well known to sensitize cells to apoptosis (157). Using normal human intestinal epithelial cells constitutively expressing a tamoxifen-responsive ER-MYC fusion protein, Yuneva et al. demonstrated that MYC expression significantly sensitized cells to apoptosis upon glutamine deprivation (132). Recent studies suggest that this phenomenon involves the up-regulation of the BH3-only proteins PUMA and NOXA (129), as well as the MYC-dependent, p53-mediated activation of the pro-apoptotic protein BAK (158).

3) *Restricted expression of pro-survival BCL-2 proteins*: Work done in our research laboratory has shown that Sp2/0 cell line expresses relatively low levels of BCL-2, BCL-xL and BCL-W (159,160). This is expected to reduce the threshold at which intrinsic apoptosis is triggered, and explains why the ectopic expression of BCL-xL is so potent at protecting Sp2/0 cells against cell death triggered by glutamine starvation and other toxic insults (140,155,159).

On the other hand, Sp2/0 cells express high levels of MCL-1. Using two inhibitors of pro-survival BCL-2 proteins (Obatoclax and ABT-737), our group demonstrated that Obatoclax (which inhibits BCL-2, BCL-xL and BCL-W and MCL-1) sensitized Sp2/0 cells to glutamine deprivation-induced apoptosis (160). On the other hand, ABT-737 (which inhibits BCL-2, BCL-xL and BCL-W but not MCL-1), was much less toxic.

1.3.2 Research Rationale

While our laboratory has shown that glutamine deprivation causes the rapid induction of apoptosis in Sp2/0 cells, how these cells sense glutamine and use that information to block the initiation of the intrinsic apoptotic pathway remains to be uncovered.

In a recent study, ammonium ions produced during glutaminolysis were shown to act as activators of autophagy, a phenomenon generally associated with cell survival (161). The authors also demonstrated that the combination of ammonium ions and α-ketoglutarate prevented the induction of apoptosis by TNF-α (161).

For the most part, the investigation of the effect of ammonium ions on cells has focused on their toxicity. This is quite understandable, considering that increased blood ammonia (for example, following chronic liver disease) is toxic to astrocytes, glial cells and neurons and can therefore lead to brain dysfunction (162). A number of studies have investigated the cellular and molecular basis for the toxicity of ammonium ions on cells. Interestingly, many of them have revealed that ammonium ions affect cell behavior through the modulation of several signaling pathways known to be involved in the cell death/cell survival response.

First, the hydrated radius of ammonium ions is very similar to K^+ ions. As a result, ammonium ions compete with the cellular uptake of potassium ions (K^+) through the Na^+/K^+-ATPase and Na^+K^+2Cl co-transporter (163-165), resulting in membrane depolarization and death. It is also worth noting that, since the efflux of K^+ ions is known to activate apoptosis (166), ammonium ions are expected to modulate this type of cell death by interfering with K^+ transport.

Secondly, ammonium ions have been shown to trigger oxidative stress, a known trigger of apoptosis (167,168). For instance, ammonium ions cause mitochondrial dysfunction (169), increase the production of free radicals (170), cause the accumulation of protein carbonyl groups (an indication of oxidative stress) (171) and lower the concentration of the reduced form of the antioxidant glutathione (GSH) (171,172).

Thirdly, ammonium ions have been observed to affect the activation status and/or the expression levels of several protein kinases involved in cell survival or cell death pathways, such as Protein Kinase C (173-175), AKT (176), and the MAPKs p38 (171,177-179) and ERK 1/2 (180).

Finally, increases in p53 cellular levels (a well-known mediator of apoptotic cell death) were demonstrated both in *vitro* (181) and in *vivo* (182) after exposure to ammonium ions.

Taken together, these observations raise the interesting possibility that ammonium ions contribute to the modulation of cell survival by glutamine.

Previously unpublished studies in our research laboratory support this idea. We observed that, when cultured in the presence of 25 µM glutamine, Sp2/0 cells underwent apoptosis but in a more protracted manner compared to cells acutely deprived of the amino acid (183). Importantly, when Sp2/0 cells cultured in the presence of 25 µM glutamine were supplemented with 5 mM of ammonium acetate or ammonium chloride, a significant and reproducible increase in cell survival was achieved. This study also indicated that when ammonium acetate was used, but not ammonium chloride, reduced caspase-3 activation. Finally, Western blot and electron microscopy data suggested that supplementation of ammonium salts to Sp2/0 cells cultured in the presence of 25 µM glutamine led to an increase in the extent of autophagy in these cells.

Considering these observations, I hypothesized that ammonium ions, a by-product of glutamine metabolism, improve the survival of glutamine-starved cells by blocking apoptosis.

To test this hypothesis, the following research objectives were pursued:

- Research objective 1: Determine the effect of ammonium ions on the survival of glutamine-starved Sp2/0 cells. To this aim, glutamine-starved Sp2/0 cells were treated with ammonium ions. Cell viability was then assessed via several complementary approaches.

- Research Objective 2: Determine the effect of ammonium ions on key components of the intrinsic apoptotic pathway in glutamine-deprived Sp2/0 cells. To this end, Sp2/0 cells which were deprived of glutamine and treated with ammonium salts were probed for the presence of morphological and biochemical apoptotic markers.

- Research Objective 3: Considering that glutamine analogs have been shown to act as a surrogate for glutamine deprivation, determine whether ammonium ions also modulate cell survival in Sp2/0 cells treated with the glutamine analogs DON and AZA.

- Research Objective 4: Considering that glutamine analogs are competitive inhibitors of glutamine-metabolizing enzymes, I sought to determine whether DON or AZA would interfere with the ability of ammonium ions to improve the viability and reduce the apoptosis of glutamine-deprived Sp2/0 cells.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Unless stated otherwise, chemicals and reagents were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Buffers and solutions were prepared using high quality H_2O, which was filtered using a Millipore Milli-Q system. The composition of all solutions in this section is described in Appendix A. A 50 x stock solution of L-glutamine (200 mM) was prepared with 1X PBS and filter-sterilized.

2.2 Cell culture

All cell culture experiments were performed in a laminar flow safety cabinet under aseptic conditions. The Sp2/0 mouse hybridoma cell line (ATCC # CRL1581) was obtained from the American Type Culture Collection (Rockville, MD). Sp2/0 cells were cultured in suspension in a humidified atmosphere at 37ºC and 5% CO_2 in Iscove's modified Dulbecco's medium (IMDM), supplemented with 5% Fetalclone I (Hyclone, Logan UT), 100 U/ml penicillin, 100 µg/ml streptomycin and 4 mM L-glutamine (hereafter referred to as complete IMDM). During routine culture, cell concentration was kept between 50,000 to 800,000 cells/ml, which corresponds to exponential growth. In all cell culture experiments, Sp2/0 cells were cultured for a maximum of four weeks after which cells were discarded to avoid the selection of mutants with undesired phenotypes. The Ramos B lymphocyte cell line (ATCC # CRL-1596) was obtained from the American Type Culture Collection and maintained in Iscove's modified Dulbecco's medium

(IMDM), supplemented with 10% Fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin solution and 2 mM L-glutamine and cultured in a humidified atmosphere at 37ºC and 5% CO_2.

2.2.1 Cryopreservation and thawing of cells

For long-term preservation, exponentially growing Sp2/0 cells were collected and resuspended in complete IMDM medium. Then, 2 x 10^6 cells were resuspended in 1 ml of cold complete IMDM medium supplemented with 10% (v/v) dimethyl sulfoxide (DMSO). Cells were frozen in cryovials overnight in a styrofoam container at -80ºC. The next day, the cryovials were transferred to a Thermolyne™ BioCane 20 cryo-container (ThermoFisher, Ottawa, ON) containing liquid nitrogen. When a new batch of cells was required, cells were retrieved from liquid nitrogen storage and rapidly thawed in a 37ºC water bath. Cells were then washed once with 10 ml of fresh pre-warmed complete IMDM to remove the DMSO. The cells were centrifuged at 1,500 rpm at 25ºC for 5 minutes, and the cell pellet was resuspended in fresh 10 ml of culture medium. Cells then transferred to 25 cm^2 cell culture flask and cultured as mentioned above.

2.2.2 Trypan blue exclusion assay.

To determine the cell number as well as cell viability in a culture, an aliquot of cells was mixed with trypan blue solution (0.4%, v/v in PBS) and left for 5 minutes at room temperature. Viable (clear) and nonviable (blue) cells were then counted using a bright-line hemocytometer. The average of at least four independent counts was used to calculate cell viability and cell number in the culture.

2.2.3 Rescue assay

To test the effect of glutamine deprivation on the survival of Sp2/0 exponentially growing cells were collected by centrifugation and washed twice with pre-warmed IMDM supplemented with 5% FetalClone I, penicillin and streptomycin (hereafter referred to as glutamine-free IMDM), resuspended in glutamine-free IMDM at a density of 400,000 cells/mL and seeded in the wells of a 24-well culture plate. Control groups were supplemented with 4mM (final concentration) of glutamine. Where indicated, cultures were supplemented with 5 mM of AA or AC with or without 1 µM of DON or 2 µM of AZA at the start of the experiment. As controls, cultures were supplemented with PBS, 5 mM sodium acetate (SA) or sodium chloride (SC). Cells were then incubated for 3 hours at which point, the cells were washed twice, resuspended in complete IMDM, and cultured for 24h. Viable and non-viable cells in each group were then counted using the trypan blue dye exclusion assay.

2.2.4 Clonogenic assay

As a complementary approach to measure cell viability, clonogenic assays were performed. In brief, cells were processed for glutamine starvation as described above. After 3h of glutamine deprivation, the cells were counted using a hemocytometer, diluted in complete IMDM to a density of approximately 5 cells /mL, plated into a 96-well plate (0.5 cells per well) and cultured at 37ºC/5% CO_2 for 10 days. Colonies consisting of more than 50 cells were then counted using light microscope.

2.2.5 Separation of viable from nonviable cells

Separation of viable from nonviable cells was performed using Ficoll-Hypaque density gradient centrifugation (184). Cells were processed for a 3h glutamine deprivation experiment as described earlier. They were then collected by centrifugation at 2,500 RPM for 5 min and resuspended in 2 ml of complete IMDM and then layered gently on 10 ml of Ficoll-Hypaque cushion in 15 ml centrifugation tube. The tubes were then centrifuged at 600 RPM for 5 min at 10ºC. After the centrifugation, the tubes were carefully removed from the rotor and, using a 1 ml micropipette, cells visible at the interface (live cells) were collected and transferred to a new 15 ml centrifugation tube. The pellet (dead cells) was also transferred into a different 15 ml centrifugation tube. Ten ml of complete IMDM was added to each tube and the cells were centrifuged for 5 min at 2,500 RPM. Finally, the supernatant was discarded, and the cells were resuspended in 2 ml of complete IMDM and counted using a hemocytometer.

2.3 Apoptosis analysis

2.3.1 Caspase 3 Colorimetric assay

Caspase-3 activity was determined using the Apoptosis Colorimetric Assay kit (Biovision, Milpitas, CA), according to the manufacturer's instructions. Briefly, $4x10^5$ cells were centrifuged at 2,500 RPM for 5 min at 25ºC and the cell pellet was resuspended in 50 µl of lysis buffer and incubated on ice for 10 min. Cells then were centrifuged at 12,000 RPM for 1 min at 4ºC then the supernatant was collected, and

protein concentration was determined using DC protein assay (Bio-Rad, Mississauga, ON). One hundred microliters of protein from each sample was transferred into a 96-well plate in triplicate. 50 µl of 2x Reaction Buffer along with 5 µl of 4 mM DEVD-pNA substrate were added to each sample and then incubated at 37ºC for 2 hours. Caspase activity was then measured at a 405 nm wavelength in a PowerWave X microplate reader (Bio-Tek, Winooski, VT). Absorbance readings were taken every 30 min for a period of 2 hours.

2.3.2 Flow cytometry analysis

Apoptotic cells were detected using flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Biovision) according to the manufacturer's protocol. Briefly, Sp2/0 cells were cultured for 2h in the presence or absence of glutamine at a density of $4x10^5$ cells/ml. After incubation, cells were harvested by centrifugation for 5 min at 2,500 RPM at 25ºC, washed once with 1x PBS and resuspended in 500 µl of 1x binding buffer. Five microliters of both Annexin V-FITC and propidium iodide (50µg/ml) were added to the cells and incubated at room temperature in the dark for 5 min. After incubation, the percentage of apoptotic cells (PI negative/Annexin V positive) and non-apoptotic cells (PI negative/Annexin V negative) was assessed by flow cytometry using a BD FACSCanto II flow cytometer and the BD FACSDiva analysis software (BD Biosciences, Mississauga, ON).

2.3.3 DNA fragmentation analysis

To assess apoptotic DNA fragmentation, 5 X 10^5 cells were harvested and washed once with PBS. Cells then were resuspended in 50 µl of lysis buffer, vortexed briefly and incubated for 5 minutes at 50ºC. Then 50 µl of loading buffer was added, and the mixture was vortexed briefly. Fifteen µl of each sample was loaded onto a 2% agarose gel and was run in TBE buffer at 95V for 45 minutes. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/ml) for 5 min, washed with H_2O and then visualized with UV transillumination using a FluorChem Imager 8000 (Alpha Innotech, San Leandro, CA).

2.3.4 Assessment of morphological changes in apoptosis

2.3.4.1 Cell staining with Hoechst 33342

Cells (4 X 10^5 / ml) were processed for glutamine deprivation as described previously. After 2.5 hours of deprivation, Hoechst 33342 was added at a final concentration of 2 µg and the culture was resumed for 30 min at 37ºC. After incubation, cells were placed on ice for 15 min and washed twice with PBS. Cells were collected by centrifugation at 2,500 RPM for 5 min at 4ºC and placed on a microscope slide and sealed with a glass coverslip. Condensed and/or fragmented nuclei were visualized with a Zeiss Axiovert 100 fluorescence microscope. Images were taken at 10x and 40x objective lenses. A minimum of 5 fields containing a total of at least 200 cells were counted.

2.4 Protein extraction and Western blot analysis

2.4.1 Whole cell protein extraction:

For each sample, 10 million Sp2/0 cells were collected by centrifugation at 2,500 RPM for 5 min at 4ºC and then washed twice with PBS and pelleted at 2,500 RPM for 5 min at 4ºC. The cell pellet was resuspended in 100 µl of RIPA lysis buffer and incubated on ice for 30 min. The mixture was then subjected to three cycles of freeze/thaw, where the samples were placed at -80ºC for 15 minutes and then immediately thawed at room temperature. The cell lysates were then placed on ice for 30 min and centrifuged at 12,500 RPM for 20 min at 4ºC. The supernatant, which contains the whole cell protein extract, was transferred into another Eppendorf tube and the protein concentration was determined using DC Protein Assay Kit (Bio-Rad) for immediate use or stored at -80ºC until needed.

For the analysis of PARP cleavage, proteins were extracted using a urea-based lysis buffer (185). The cell pellets were resuspended in the urea-based lysis buffer, subjected to sonication on ice for three intervals of 15 s using a Model 50 Sonic Dismembrator (Thermo-Fisher). After centrifugation (12,500 RPM, 20 min), the supernatant was collected and stored at -80ºC until needed. The protein extracts were heated for 15 min at 65ºC before being subjected to PAGE-SDS electrophoresis.

2.4.2 Cytosolic protein extraction

Ten million Sp2/0 cells were collected and placed on ice for 15 min, pelleted at 2,500 RPM for 5 min at 4ºC and then washed twice with ice-cold PBS. The cell pellets were resuspended in 250 µl of buffer A and placed on ice for 10 min. Cell lysis was assessed by staining cells with trypan blue. Cells were then centrifuged at 12,000 RPM for 5 min at 4ºC, and the supernatant was recovered and centrifuged again under the same conditions. Following centrifugation, the supernatant was recovered, 1 volume of 2x RIPA lysis buffer was added, and this was used as the cytosolic fraction. Protein concentration was determined using DC Protein Assay kit and immediately processed for Western blotting or stored at -80ºC until needed.

2.4.3 Determination of protein concentration

The DC Protein Assay Kit was used according to the manufacturer's instructions to measure the protein concentration in total or cytosolic protein extracts. Bovine serum albumin (BSA) standards (0.25 mg/ml - 2.0 mg/ml) were used to generate a standard curve. Each sample of unknown protein concentration was diluted to a ratio of 1:10 in RIPA lysis buffer. Five microliters of each BSA standard and sample was loaded in triplicate in the wells of a 96 well plate and 25 µl of reagent A and 200 µl of reagent B were added respectively. The mixture was incubated at room temperature for 15 min, then, the absorbance at 750 nm of each well was read using a Bio-Tek PowerWave-X microplate reader.

2.4.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Tris-glycine)

Protein separation was performed using 10% SDS-PAGE gel. Fifty micrograms of protein lysate from each sample in 1x sample buffer was boiled for 5 min, placed on ice for 2 min and then briefly centrifuged at 2,500 for 10 seconds at room temperature. The samples were then subjected to fractionation by electrophoresis using Bio-Rad Mini-Protean II apparatus in 1x Tris-Glycine running buffer and run at 150 V for 90 min. A Precision Plus Protein Dual color ladder (BioRad) was also loaded onto the gel to confirm protein sizes. After that, gels were removed from the unit and placed in a transfer buffer for 5 min. The resolving and stacking gel compositions are listed below in table 2-1.

Table 2-1 Composition and volumes for SDS-PAGE gels

Reagent	Separating Gel			Stacking Gel
	10%	12%	15%	
30% Acrylamide (ml)	5.0	6.0	7.5	0.65
Tris/SDS pH 6.8 (ml)	--	--	--	1.25
4Xtris/SDS pH 8.8 (ml)	3.75	3.75	3.75	--
dd H_2O (ml)	6.25	5.25	3.75	3.05
TEMED (µl)	10	10	10	5
10% APS (µl)	50	50	50	25

2.4.5 Tris-Tricine SDS-PAGE

Tris-Tricine SDS-PAGE was used to separate proteins smaller than 20 kDa in size.

Protein samples were prepared as described above. A separating gel contained 12% Tris-tricine SDS-PAGE. The stacking gel contained 3.89% acrylamide and 2x Tris-Cl/SDS loading buffer. The gel was run at 30 mA for 3-5 h in a Mini-Protean II electrophoresis unit, and cathode buffer and anode buffer were used as running buffers. Below is the composition of separating and stacking gels in table 2-2.

Table 2-2 Composition of Separating and Stacking Gels in Tricine SDS-PAGE

Reagent	Separating Gel	Stacking Gel
30 % Acrylamide (ml)	5.9	0.81
Tris·HCl/SDS, pH8.45 (ml)	5	1.55
H_2O (ml)	0.515	3.89
Glycerol 50% (ml)	4	--
10% APS (µl)	50	25
TEMED (µl)	20	10

2.4.6 Western blot analysis

Proteins separated by SDS-PAGE were transferred to an Immobilon-P Polyvinylidene fluoride (PVDF) membrane (Millipore, Etobicoke, ON). All membrane transfers were performed using a TransBlot semi-dry transfer apparatus (Bio-Rad). The PVDF membrane was immersed in 100% methanol for 15 seconds then presoaked in transfer buffer for 5 min. 3 MM Filter papers were cut to the same size as the PVDF membrane and also soaked in transfer buffer for 5 min. Protein transfer was set to run for 45-60 min at 15 volts. After the transfer was completed, the PVDF membrane was stained with Ponceau S for 5 min to determine the transfer efficiency and to

ensure that equal loading had occurred. The gel was also stained with Coomassie Brilliant Blue R-250 dye for a minimum of 30 min then placed in de-staining buffer until the background became clear. The PVDF membrane was quickly rinsed with water to remove the stain and immediately placed in 5% blocking buffer in TTBS buffer with gentle agitation for 1 h at room temperature. Primary antibodies were diluted in 5% blocking buffer at the indicated concentrations (Table 2-3) and then incubated with gentle shaking overnight at 4ºC. The next day, the membrane was washed three times 5 min in TTBS at room temperature and incubated with anti-Rabbit Horseradish Peroxidase-conjugated secondary antibody (Cell Signaling Technology) diluted at 1:3000 in blocking buffer for 1 h at room temperature. The membrane was washed in TTBS as previously described and then incubated with chemiluminescent Immobilon Western HRP substrate (Millipore) for 5 min at room temperature. The chemiluminescent signal was finally captured using a Fluorochem 8000 Imaging System.

Table 2-3 List of primary antibodies used in SDS-PAGE immunoblotting

Antibody	Company and catalog #	Final concentration	Incubation conditions
β-Actin	Cell Signaling-4967	1:1000 dilution	O/N at 4ºC
Caspase-3	Cell Signaling-9662		
Cytochrome c	Cell Signaling-4272		
HSP60	Cell Signaling-4869		
Lamin A/C	Cell Signaling-2032		
PARP	Cell Signaling-9542		

2.4.7 Densitometry

Densitometric analysis of the intensity of selected protein bands was performed using the AlphaEase software (Alpha Innotech). To measure the percentage of each protein band, level of loading control was used as a base for any variation in band intensity.

2.5 Statistical analysis

All experiments were independently performed at least three times on different days. Statistical significance of differences between study and control groups was determined by one-way ANOVA (Microsoft Excel^{TM}) where *p* values below 0.05 were considered statistically significant.

3. RESULTS

3.1 Effect of ammonium ions on cell viability

Our research team has previously shown that Sp2/0 cells display apoptotic biochemical features a few minutes only after being deprived of glutamine (131). Considering that glutamine-derived ammonium ions have been shown to exhibit pro-survival properties (161), I investigated whether the supplementation of ammonium ions could modulate the susceptibility of Sp2/0 cells to undergo apoptosis upon glutamine deprivation.

3.1.1 Ammonium ions improve cell viability upon glutamine starvation

I first tested the effect of ammonium ions on the viability of Sp2/0 cells in a 3h glutamine-deprivation rescue assay. In glutamine-deprived cultures supplemented with PBS, Sp2/0 cell viability was approximately 30%, which is similar to previously reported results from our group (131) (Figure 3-1). Interestingly, when glutamine-starved cultures were supplemented with 5mM ammonium acetate (AA) or ammonium chloride (AC), cell viability increased to 70% and 54%, respectively. Importantly, glutamine-deprived cultures supplemented with 5 mM sodium acetate (SA) or sodium chloride (SC) behaved similarly to the PBS-treated control. Finally, the fact that the ammonium ion/glutamine-supplemented groups showed cell viabilities similar to the PBS control indicates that, under our experimental conditions, ammonium ions did not have a toxic effect on the cells (Figure 3-1). As is shown in Figure 3-2, the optimal protective effect of ammonium ions on glutamine-starved Sp2/0 was found to be 5mM.

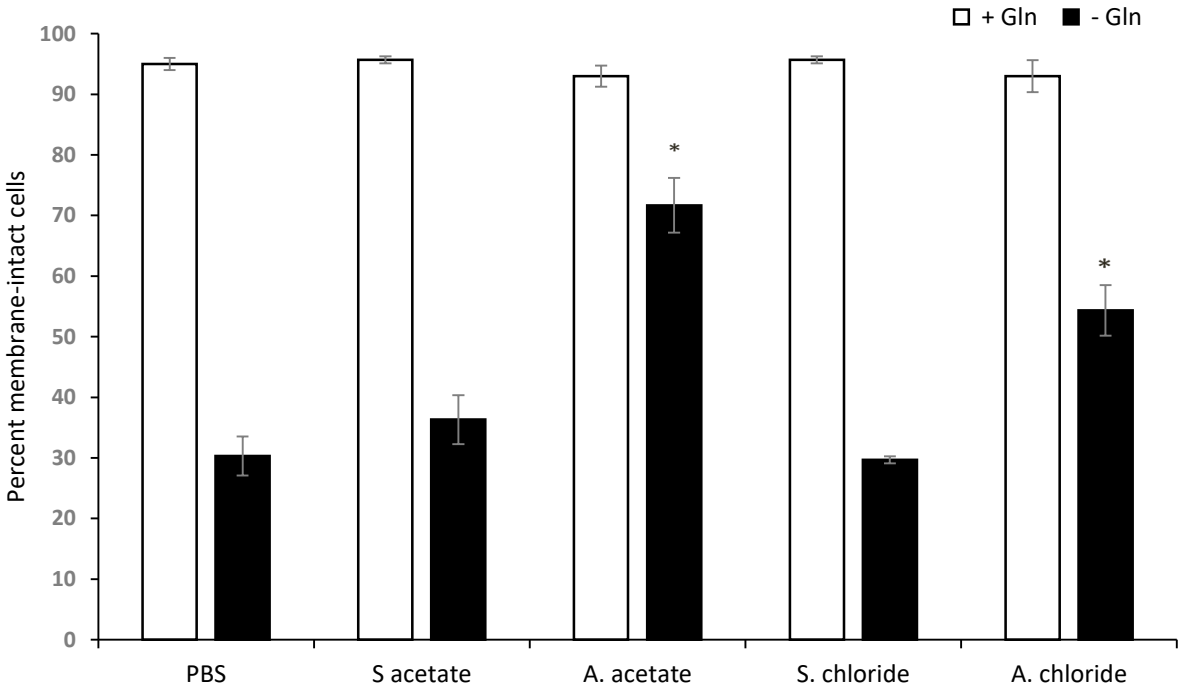


Figure 3-1 Ammonium salts improve the viability of Sp2/0 cells cultured in Gln-starved medium.

Cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Gln (4 mM) was then added and culture was resumed for 24 h. Cell viability was determined by trypan blue exclusion. Cultures in which Gln was added at the start of the experiment were included as controls. Data are the average ± SD of 3 independent experiments. * $p<0.05$ vs corresponding sodium salt- supplemented control. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

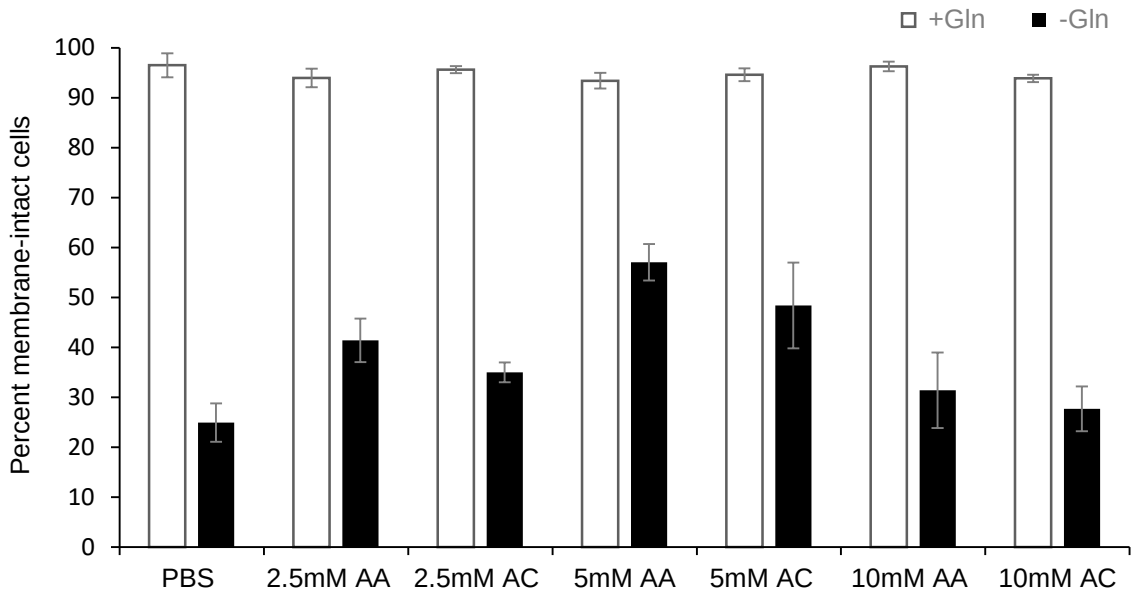


Figure 3-2 Dose response of different concentrations of ammonium salts on cell viability

Sp2/0 cells were deprived of glutamine (Gln) for 3h in the presence of the concentrations stated above for ammonium acetate and ammonium chloride or an equivalent volume of PBS. Gln (4 mM) was then added and culture was resumed for 24 h. Cell viability was determined by trypan blue exclusion. Cultures in which Gln was added at the start of the experiment were included as controls. Data are the average ± SD of 3 independent experiments. SC: sodium chloride. SA: sodium acetate. AC: ammonium chloride. AA: ammonium acetate.

It is important to note that the cytoprotective effect of ammonium ions was not achieved when 5 mM of methylamine chloride, a structurally similar compound to ammonia, was supplemented to glutamine-starved Sp2/0 cells for 3 hours in a rescue assay, indicating that the pro-survival effect is specific to ammonium ions (Figure 3-3).

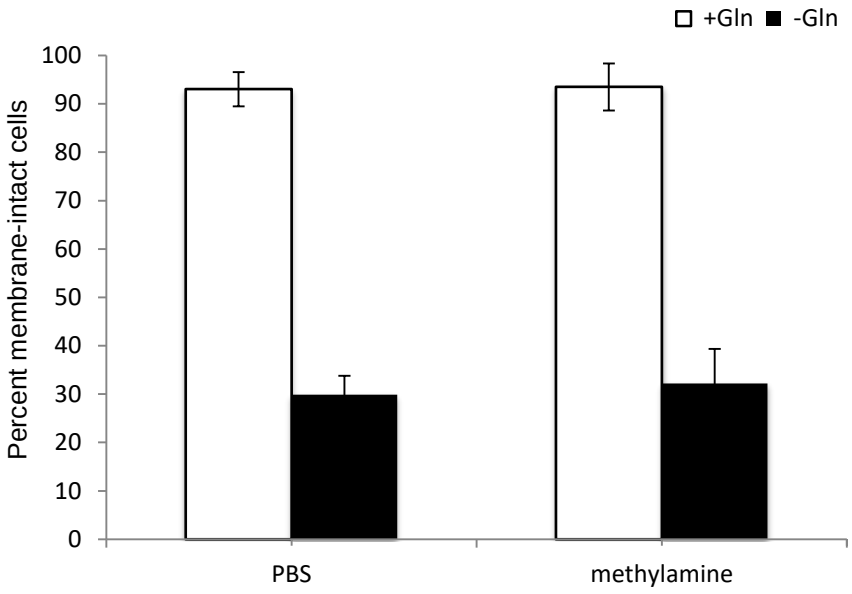


Figure 3-3 Methylamine has no effect on cell viability

Sp2/0 cells were deprived of glutamine for 3h in the presence of 5 mM of methylamine or an equivalent volume of PBS. Gln (4 mM) was then added and culture was resumed for 24 h. Cell viability was determined by trypan blue exclusion. Cultures in which Gln was added at the start of the experiment were included as controls. Data are the average ± SD of 3 independent experiments.

3.1.2 Ammonium salts improve cell viability in the presence of Glutamine analogs

I first examined the effect of glutamine analogs on Sp2/0 cell viability. Sp2/0 cells were incubated for 24 hours in complete IMDM containing glutamine with 1 µM DON or 2 µM AZA alone and control groups were supplemented with PBS. Cell viability was assessed by trypan blue exclusion assay. Figure 3-4 shows that, while the viability of the control group was over 90%, Sp2/0 cells treated with DON or AZA had almost 80% dead cells. This confirms that interfering with glutamine-metabolizing pathways is sufficient to trigger cell death in this cell line, even in the presence of glutamine.

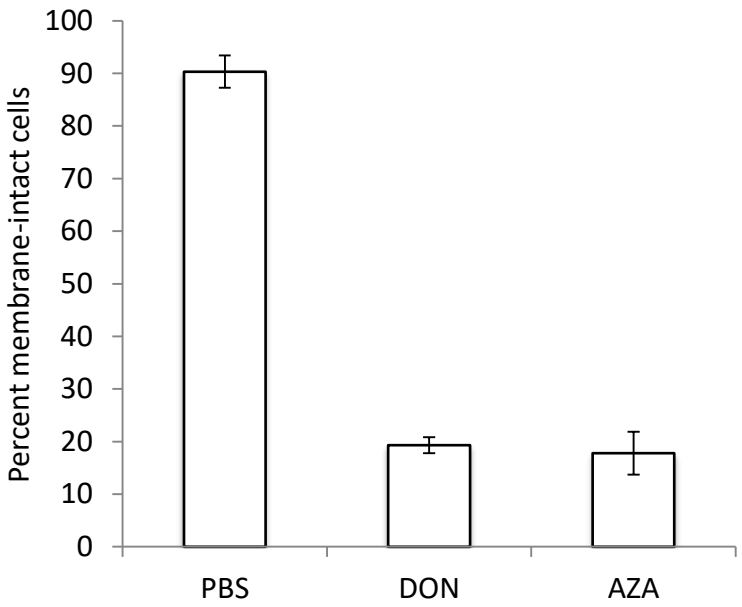


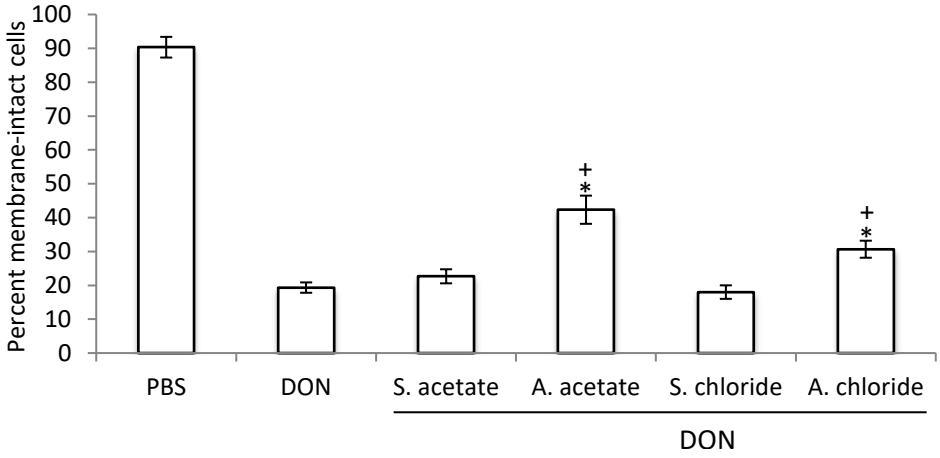
Figure 3-4 Glutamine-analogs, AZA or DON cause cell death in Sp2/0 despite the presence of glutamine.

Cells were cultured with glutamine for 24h in the presence of 1µM of DON or 2µM of AZA which were added at the start of the experiment. Cell viability was determined by trypan blue exclusion. Data are the average ± SD of 3 independent experiments. DON: 6-Diazo-5-oxo-L-norleucine, AZA: azaserine.

I next tested the effect of ammonium ions on the viability of Sp2/0 cells treated with DON or AZA in the presence of glutamine. Sp2/0 cells were cultured for 24 h in complete IMDM in the presence of 2 µM AZA or 1 µM DON and 5 mM of AA or AC. Control groups were supplemented with PBS or the corresponding sodium salt. When Sp2/0 cells were treated with the combination of SC and DON or SA and DON a reduction in viability similar to the one observed in the presence of the glutamine analog alone was observed (Figure 3-5A). Interestingly, when cells were cultured for 24 h in the presence of DON and either AA or AC, the viability was significantly higher compared to controls. Similar results were obtained when AZA was used (Figure 3-5B). Thus, the

sensitivity of Sp2/0 cells to glutamine analogs can be decreased by the supplementation with ammonium salts.

A)



B)

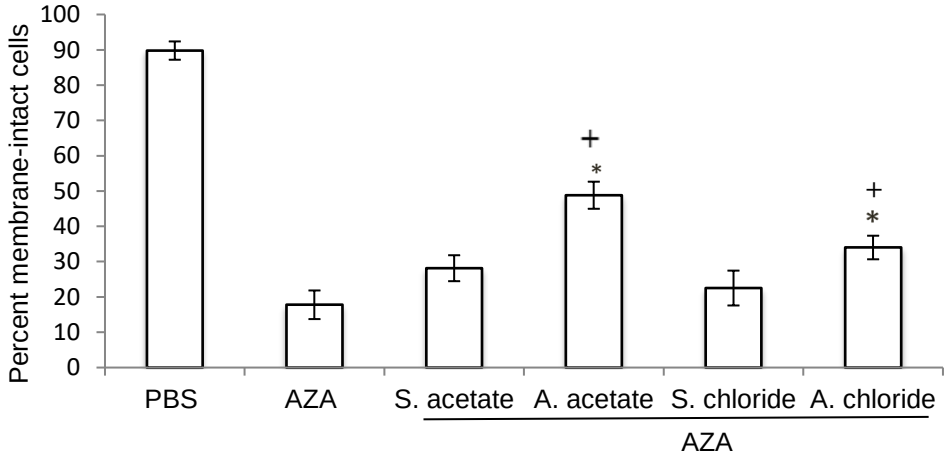


Figure 3-5 Ammonium salts improve the viability of Sp2/0 in the presence of DON or AZA.

Cells were cultured with glutamine for 24h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. 1µM of DON or 2µM of AZA were added at the start of the experiment. Cell viability was determined by trypan blue exclusion. Data are the average ± SD of 4 independent experiments. *$p<0.05$ vs. DON or AZA. $^{+}p<0.05$ vs. corresponding sodium salt- supplemented control. DON: 6-Diazo-5-oxo-L-norleucine, AZA: azaserine, S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

The above observations raised a question whether this protection against apoptosis, exerted by ammonia, is transient or whether it would lead to long-lived cells capable of proliferation. To address this question, I treated Sp2/0 cells with 5 mM of AA, AC, SA, SC and PBS for 3 hours in the presence or absence of glutamine and then processed them for a clonogenic assay. As seen in Figure 3-6, glutamine-starved cells treated with either AA or AC showed a significant increase in colony formation compared to the control groups, confirming that ammonium ions modulated the viability and preserved the proliferative capacity of glutamine-deprived Sp2/0 cells.

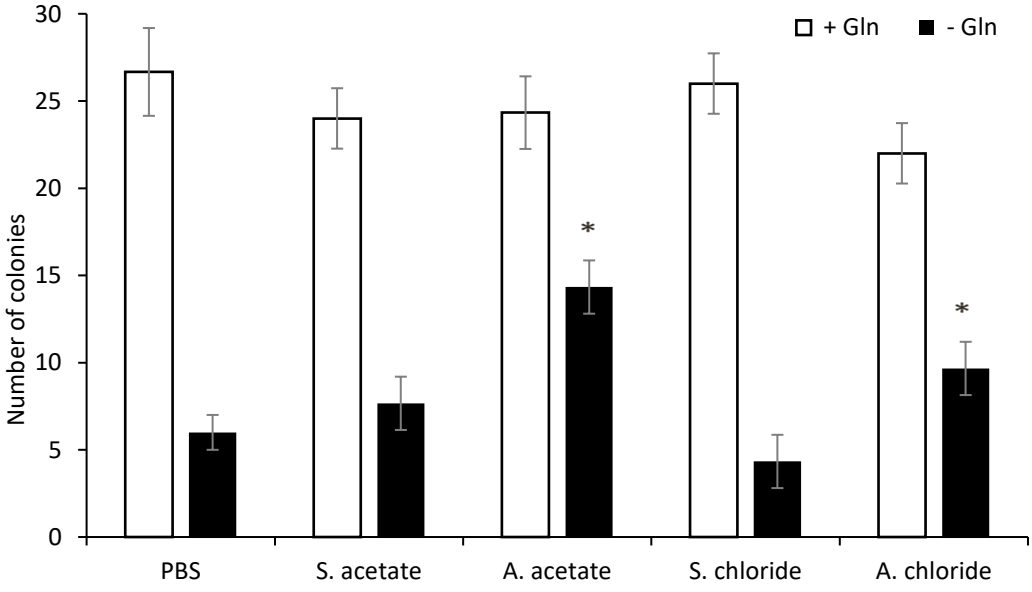
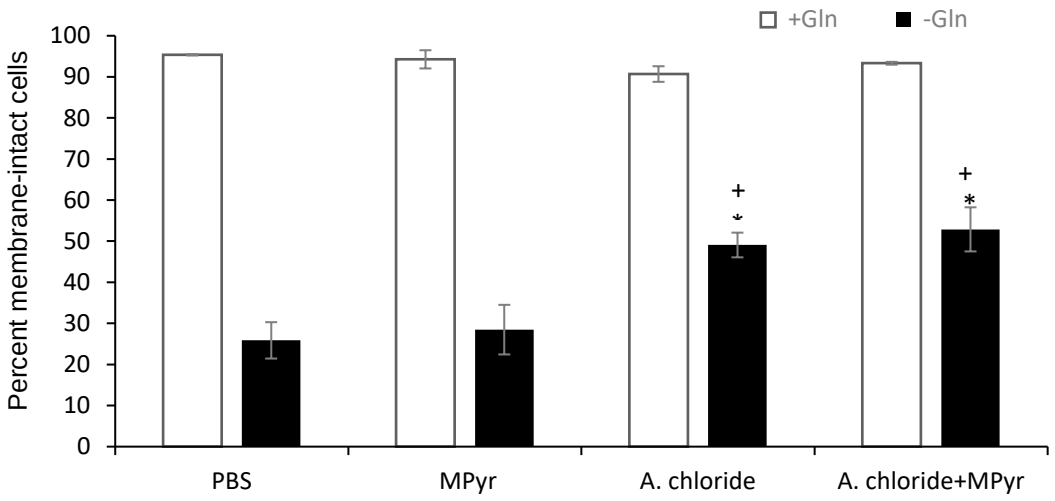


Figure 3-6 Ammonium ions improve Sp2/0 cells proliferation in clonogenic assay

Cells were deprived of glutamine for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Cells were then processed for a clonogenic assay. Data are the average ± SD of 3 independent experiments. * $p<0.05$ vs. corresponding sodium salt- supplemented control. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

When I looked at the data obtained, I noticed that glutamine-starved cells exposed to AA showed a greater cell viability compared to cells supplemented with AC (Figure 3-1, Figure 3-6). Therefore, I asked the question whether acetate contributes to the effect of ammonium salts by acting as a carbon source that can be used by the cell to maintain its metabolism. To address this question, I tested the effect of methyl pyruvate (MePyr), a membrane-permeant pyruvate analog, and glutamate, which are both substrates for the TCA cycle and ATP production, on the viability of glutamine-starved Sp2/0 cells. Therefore, I cultured Sp2/0 cells with or without glutamine for 3 hours and supplemented with 5 mM of AC in the presence or not of 4 mM MePyr.

A)



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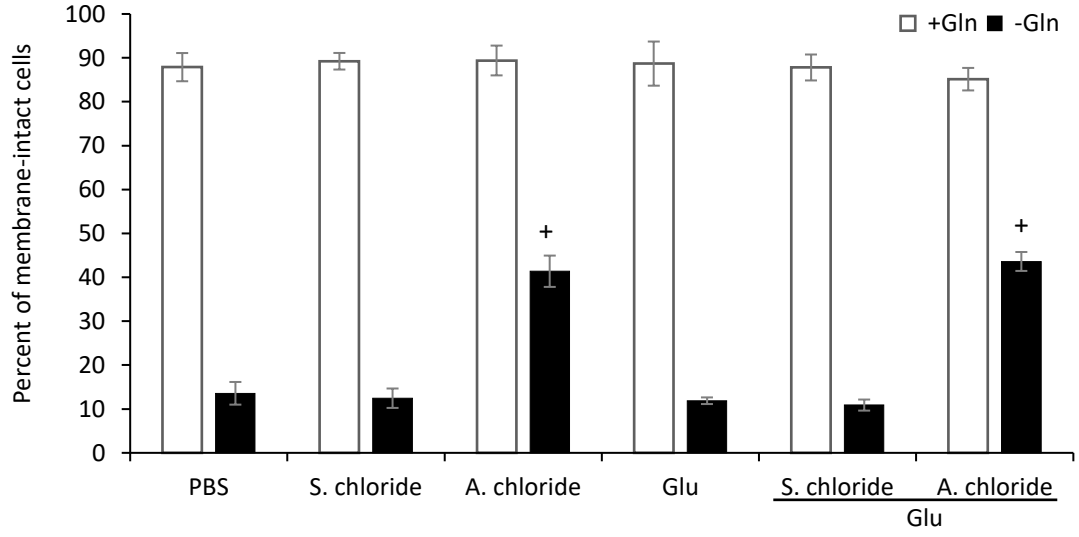


Figure 3-7 Methyl pyruvate and glutamate have no effect on cell viability

A) Sp2/0 cells were treated with 5 mM of each of the indicated compounds or an equivalent volume of PBS for 3 hours in the presence or absence of glutamine (Gln). The cells were then processed for the determination of viability by the rescue assay. B) A similar experiment was performed as in panel A. except that glutamate was used instead of MePyr. Data are the average ± SD of 3 independent experiments. * $p<0.05$ vs. PBS**.** + $p<0.05$ vs. corresponding sodium salt- supplemented control**.** S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

I observed that the viability of cells treated with AC was significantly higher than cells treated with MePyr or PBS. However, the combination of AC with MePyr did not lead to a greater increase in the viability of glutamine-starved cells compared to the cells treated with AC alone (Figure 3-7A). Similar results were obtained when glutamate was used instead of MePyr (Figure 3-7B). These results suggest that the greater improvement of cell viability by AA is not due to presence of acetate as a carbon source.

3.1.3 Non-additive effect of ammonium salts and α -ketoglutarate on the viability of glutamine-starved Sp2/0 cells

To further investigate the effect of glutamine deprivation on cell viability, I investigated the role of α-ketoglutarate, another product of glutaminolysis. A rescue assay was performed where Sp2/0 cells were cultured in complete IMDM with and without glutamine and supplemented with 1 mM of α-ketoglutarate alone or in combination with AA, AC, SA or SC for 3 hours. The results show that α-ketoglutarate alone significantly improved cell viability in the absence of glutamine; however, there was no significant increase in viability when α-ketoglutarate was added with ammonium salts. This shows that there was no additive effect of α-ketoglutarate and ammonium salts on the viability of glutamine-starved Sp2/0 cells (Figure 3-8). These data suggest that the effect of ammonium ions on Gln-starved Sp2/0 cells viability is not the result of increased glutamine synthesis.

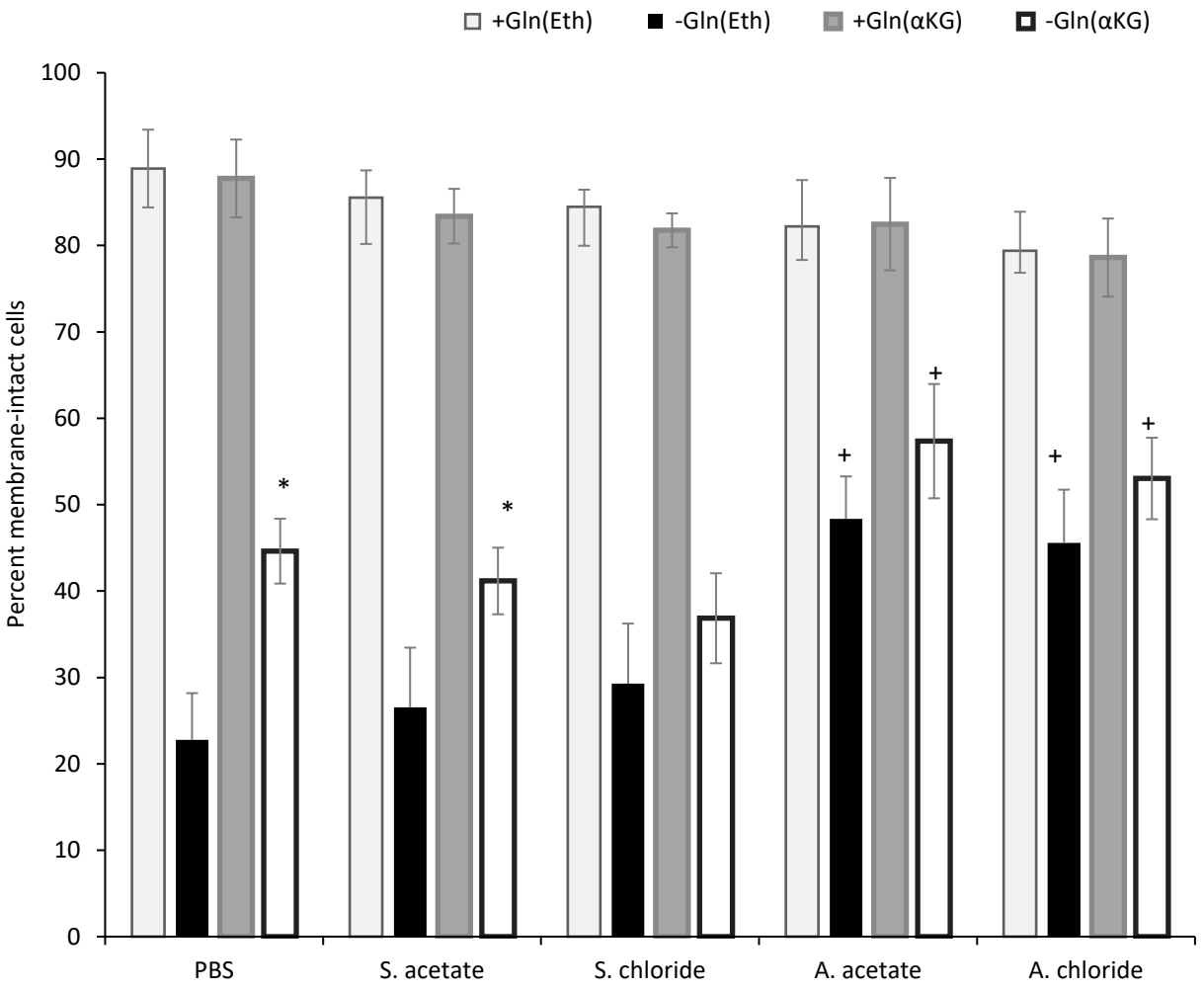


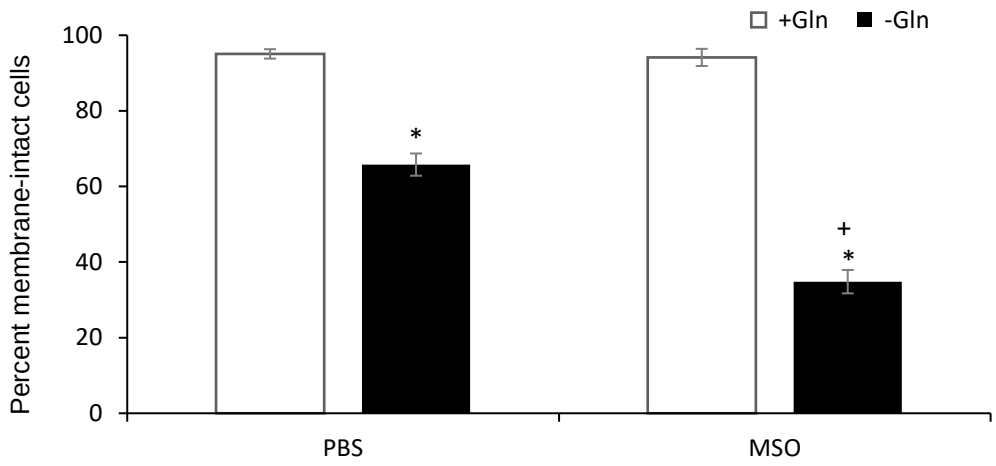
Figure 3-8 Non-additive effect of ammonium salts and α-ketoglutarate on the viability of glutamine-starved Sp2/0 cells

Cells were deprived of glutamine for 3h in the presence of 2 mM dimethyl α-ketoglutarate (αKG) and 5 mM ammonium/sodium salt or an equivalent volume of PBS. Cultures in which the solvent ethanol (Eth) was added in place of αKG were included as controls. Gln (4 mM) was then added and culture was resumed for 24 h. Cell viability was determined by trypan blue exclusion. Cultures in which Gln was added at the start of the experiment were included as controls. *$p<0.05$ vs. corresponding ethanol-supplemented control. +$p<0.05$ vs. corresponding sodium salt-supplemented control. Data are the average ± SD of 4 independent experiments. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

3.1.4 Effect of ammonium salts on the viability of glutamine-starved Sp2/0 cells does not depend on glutamine synthesis

Ammonium ions are produced during glutamine metabolism by conversion of glutamine to ammonia and glutamate by the enzyme GLS (186). Glutamine can be generated by an opposite reaction from glutamate and ammonium ions by the enzyme glutamine synthetase (GS) (90,187,188). Thus, I asked if the promotion of cell viability by ammonium ions was the result of increased glutamine production via GS. To answer this question, I used the GS inhibitor Methionine Sulfoximine (MSO) and again assessed cell viability during a glutamine-starvation rescue experiment. To confirm that MSO was functional, I first tested MSO on Ramos Burkitt's lymphoma cells, a MYC-overexpressing cell line (189). The viability of Ramos cells deprived of glutamine for 24 hours was significantly reduced in the presence of 10 mM of MSO (Figure 3-9A). However, when supplemented to glutamine-starved Sp2/0 cultures, MSO had no effect on cell viability (Figure 3-9B). The lack of effect of MSO on Sp2/0 cells is not surprising as Sp2/0 cells have been shown to express low levels of GS (131,190).

A)



B)

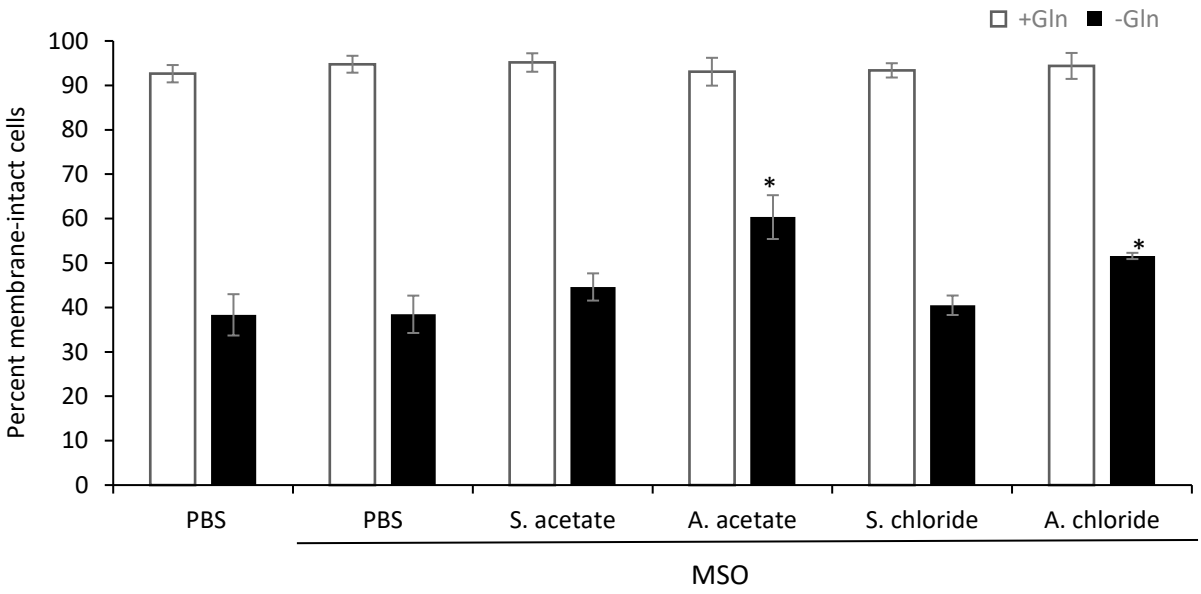


Figure 3-9 MSO treatment has no effect on cell viability

A) Ramos cells were deprived of L-glutamine (Gln) for 24 h in the presence or absence of MSO. Data are average ± SD of 3 independent experiments. *p<0.05 vs. corresponding control cultured in the presence of Gln. +p< 0.05 vs corresponding PBS control. B) Sp2/0 cells were deprived of Gln for 3 h in the presence of MSO and 5 mM ammonium/ sodium salt or an equivalent volume of PBS. Gln (4 mM) was then added and culture was resumed for 24 h. Cell viability was determined by trypan blue exclusion. For both panels, cultures in which Gln was added at the start of the experiment were included as controls. Data are the average ± SD of 3 independent experiments.* p<0.05 vs. corresponding sodium salt-supplemented control. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate**.**

3.1.5 Effect of ammonium ions on apoptosis

3.1.5.1 Cell morphology

I established so far that treatment of glutamine-deprived Sp2/0 cells with ammonium ions led to a significant increase in viability. I next asked whether this phenomenon was due to an effect of ammonium ions on the apoptotic machinery. To address this, I first examined the effect of ammonium salt supplementation on the morphology of glutamine-deprived Sp2/0 cells. While the glutamine-starved cells supplemented with PBS or the sodium salts showed typical apoptotic features (e.g. smaller cell size, apoptotic body formation) treatment with either AA or AC led to a clear increase in the number of cells with an apparent normal morphology (Figure 3-10). Thus, these data suggested that ammonium salts interfered with apoptotic processes.

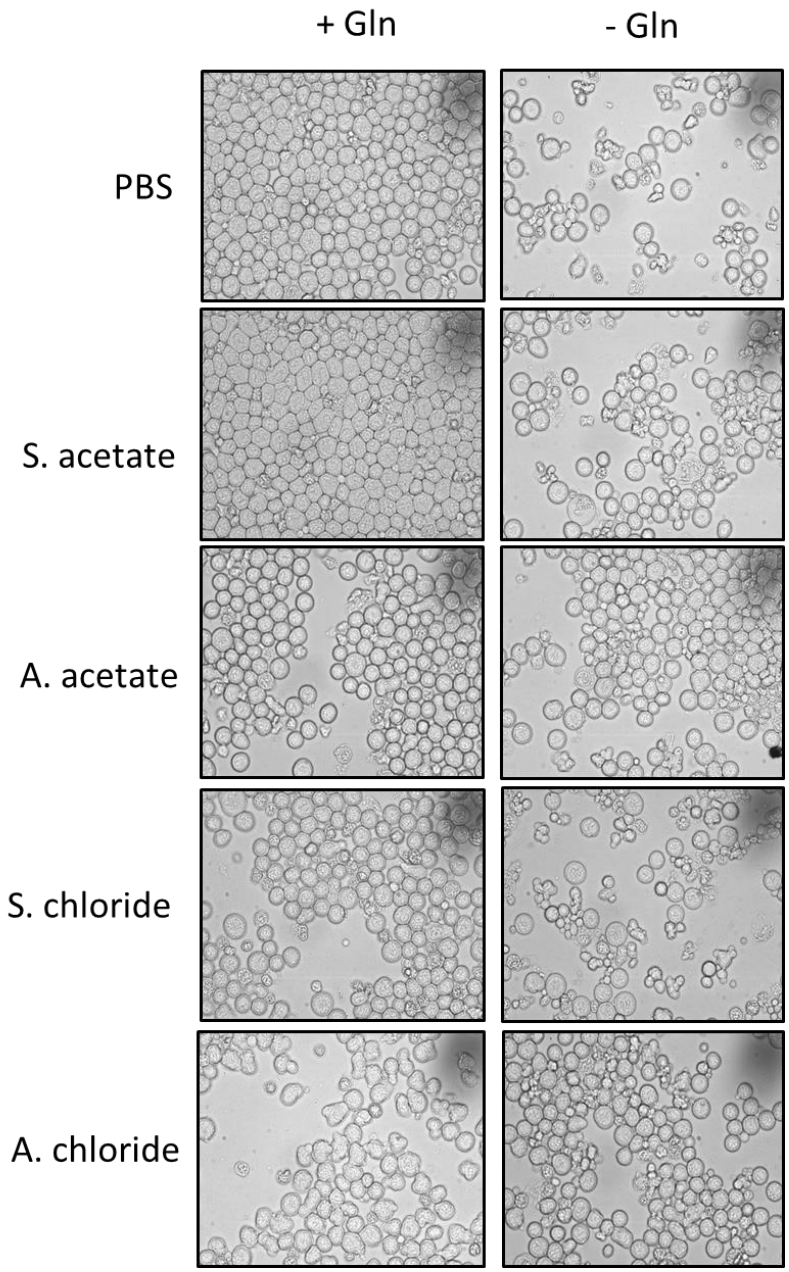


Figure 3-10 Ammonium ions improve the morphology of glutamine- starved Sp2/0 cells.

Cells were deprived of glutamine (- Gln) for 3 h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Cultures in which Gln was added at the start of the experiment (+ Gln) were included as controls. Magnification: 400x. S**.** chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

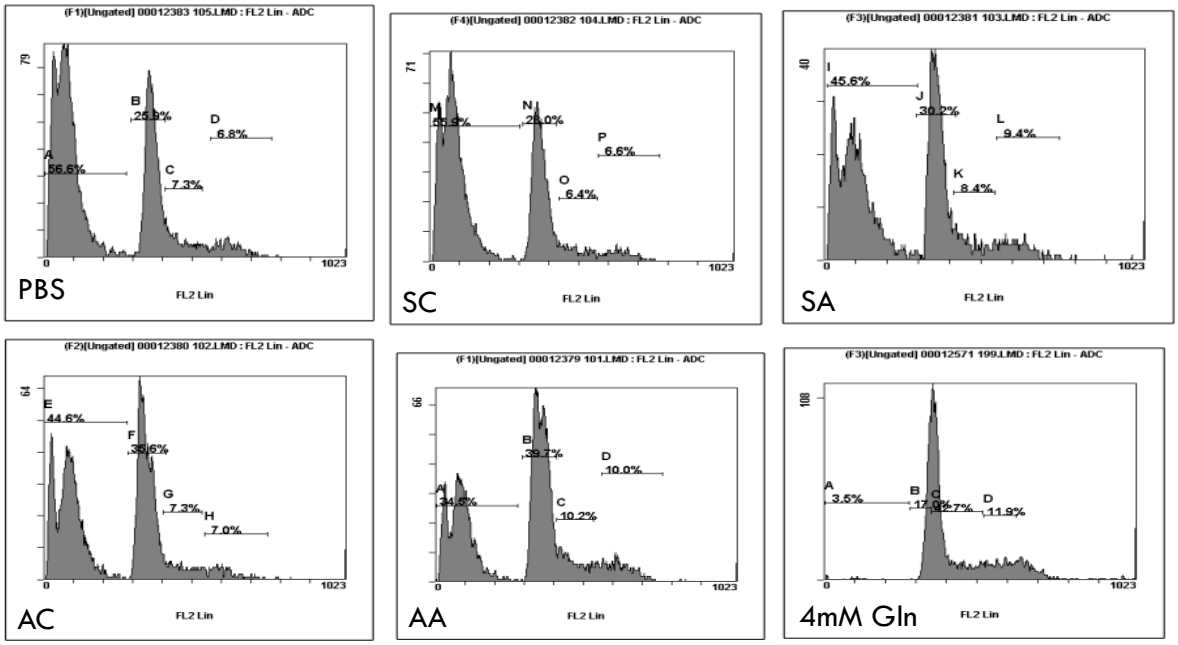
3.1.5.2 Ammonium ions lower Gln-starved Sp2/0 cell in sub-G phase

One of the hallmarks of apoptosis is cell fragmentation due to the formation of apoptotic bodies. The latter can easily be detected by flow cytometry as a sub-G1 peak on DNA content frequency histogram. Thus, I used flow cytometry analysis of the DNA content of glutamine-starved Sp2/0 cells to confirm the effect of ammonium ions on apoptotic processes. Our results showed that the percentage of sub-G1 events in Sp2/0 samples deprived of glutamine and supplemented with sodium acetate, sodium chloride or PBS was 45.6%, 55% and 56%, respectively (Figure 3-11). The percentage of sub-G1 events in AC-supplemented cells was not different from the controls, at 44.6%. However, the percentage of the sub-G1 event in the glutamine-deprived, AA-treated cells was significantly lower, at 34.5%. Altogether, these results suggest that AA, but not AC, delayed the progression of apoptosis in glutamine-starved Sp2/0 cells.

3.1.5.3 Detection of Apoptosis using Annexin-V and Propidium Iodide staining

I next examined PS externalization, an apoptotic event which can be easily detected by flow cytometry by Annexin-V (AnnV)-FITC staining (191). Co-staining the cells with propidium iodide (PI) staining allows the distinction between early apoptotic cells (AnnV positive / PI-negative) from late apoptotic cells (AnnV positive/PI positive).

A)



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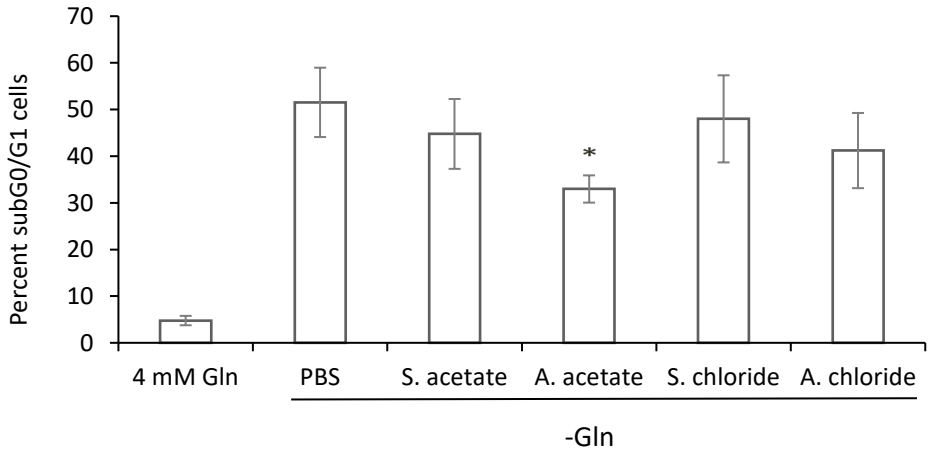


Figure 3-11 Ammonium salts lower Sub-G1 Sp2/0 cells.

A)Sp2/0 cells were cultured with or without glutamine and treated with 5 mM of ammonium/sodium salt or an equivalent volume of PBS for 3 hours and analyzed by flow cytometry for DNA content as an indication of apoptosis. B) A histogram representation of the percentage of non-apoptotic cells in the sub-G1 stage of cell cycle. Data are the average ± SD of 3 independent experiments. *p<0.05 vs. PBS. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

The results shown in Figure 3-12 and Figure 3-13 clearly indicated that supplementation of glutamine-starved Sp2/0 cultures with either AA or AC led to a significant increase in the number of viable (AnnV negative/PI negative) cells when compared to the groups supplemented with PBS or the corresponding sodium salts. Thus, ammonium ions modulate a common marker of apoptosis in glutamine-starved Sp2/0 cells.

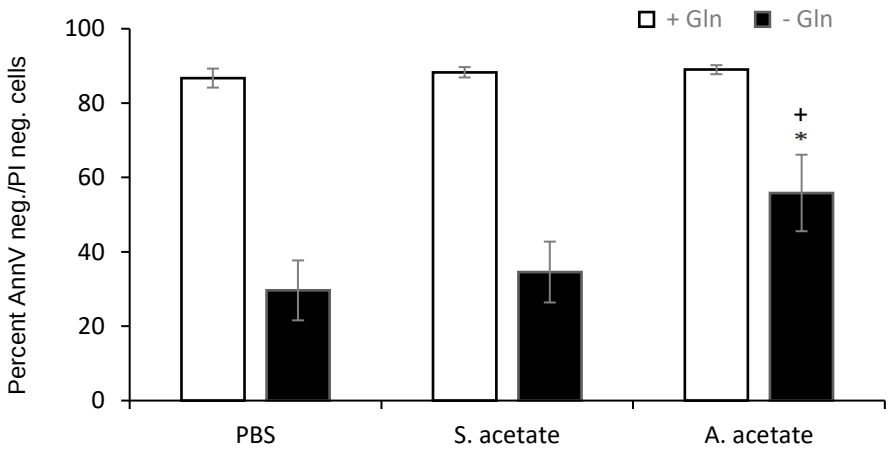


Figure 3-12 Ammonium salts improve the viability of glutamine-starved Sp2/0 cells –flow cytometry assays (AA).

Cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Cells were then processed for the determination of cell viability by flow cytometry. A histogram representation of the percentage of live and apoptotic cells. Data are the average ± SD of 3 independent experiments. . *p<0.05 vs PBS. +p<0.05 vs. corresponding sodium salt-supplemented control. S. acetate: sodium acetate. A. acetate: ammonium acetate. Representation of raw data is presented in Appendix D. Viable, early/late apoptotic and necrotic cells distribution is presented in Appendix C.

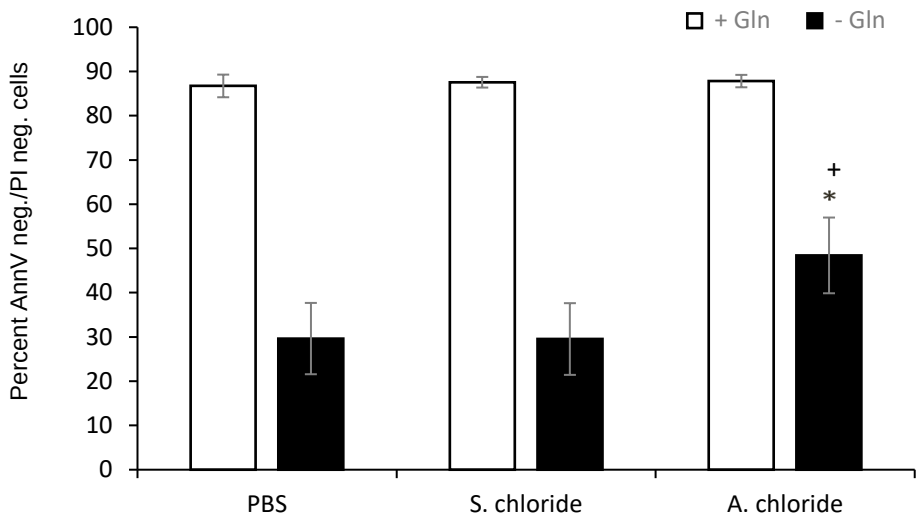


Figure 3-13 Ammonium salts improve the viability of glutamine-starved Sp2/0 cells –flow cytometry assays (AC).

Cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Cells were then processed for the determination of cell viability by flow cytometry. A histogram representation of the percentage of live cells. Data are the average ± SD of 3 independent experiments. .*$p<0.05$ vs PBS. +$p<0.05$ vs. corresponding sodium salt-supplemented control. S. chloride: sodium chloride. A. chloride: ammonium chloride. Representation of raw data is presented in Appendix E. Viable, early/late apoptotic and necrotic cells distribution is presented in Appendix C.

3.1.5.4 Ammonium salts do not prevent caspase activation

I next investigated the possibility that ammonium ions interfered with core apoptotic processes in glutamine-starved Sp2/0 cells. As a first step, I examined the effect of ammonium ions on caspase activation. To this end, Sp2/0 cells were deprived of glutamine for 3h in the presence of ammonium salts, PBS, or the corresponding sodium salt control. The cells were then processed for a caspase-3 activity assay. The results

obtained indicate that, as expected, glutamine starvation led to a significant increase in caspase-3 activity over the glutamine-supplemented controls, irrespective of whether the cultures were supplemented with PBS or the sodium salts (Figure 3-14D). Surprisingly, however, culture supplementation with either AA or AC did not prevent caspase-3 activation. This result was confirmed when I examined caspase-3 processing from its inactive 33 kDa precursor into its 17 and 19 kDa subunits (Figure 3-14E) and found that culture supplementation with ammonium salts did not impede the cleavage of procaspase-3 into its active, processed form. Finally, I analyzed the cleavage of a number of caspase-3 substrates: poly (ADP-ribose) polymerase (PARP) (Figure 3-14B), lamin A/C (Figure 3-14A), as well as DNA fragmentation (Figure 3-14C) (the latter being the result of the caspase-3-mediated inactivation of ICAD, an inhibitor of the caspase-activated DNAse). In all three of these instances, culture supplementation with ammonium salts did not prevent cleavage of lamin A/C, PARP nor DNA fragmentation (Figure 3-14A/B/C). All in all, these data indicate that the supplementation of glutamine-starved Sp2/0 cells with ammonium salts, at concentrations that led to a significant improvement in cell survival, does not prevent the activation of caspase-3.

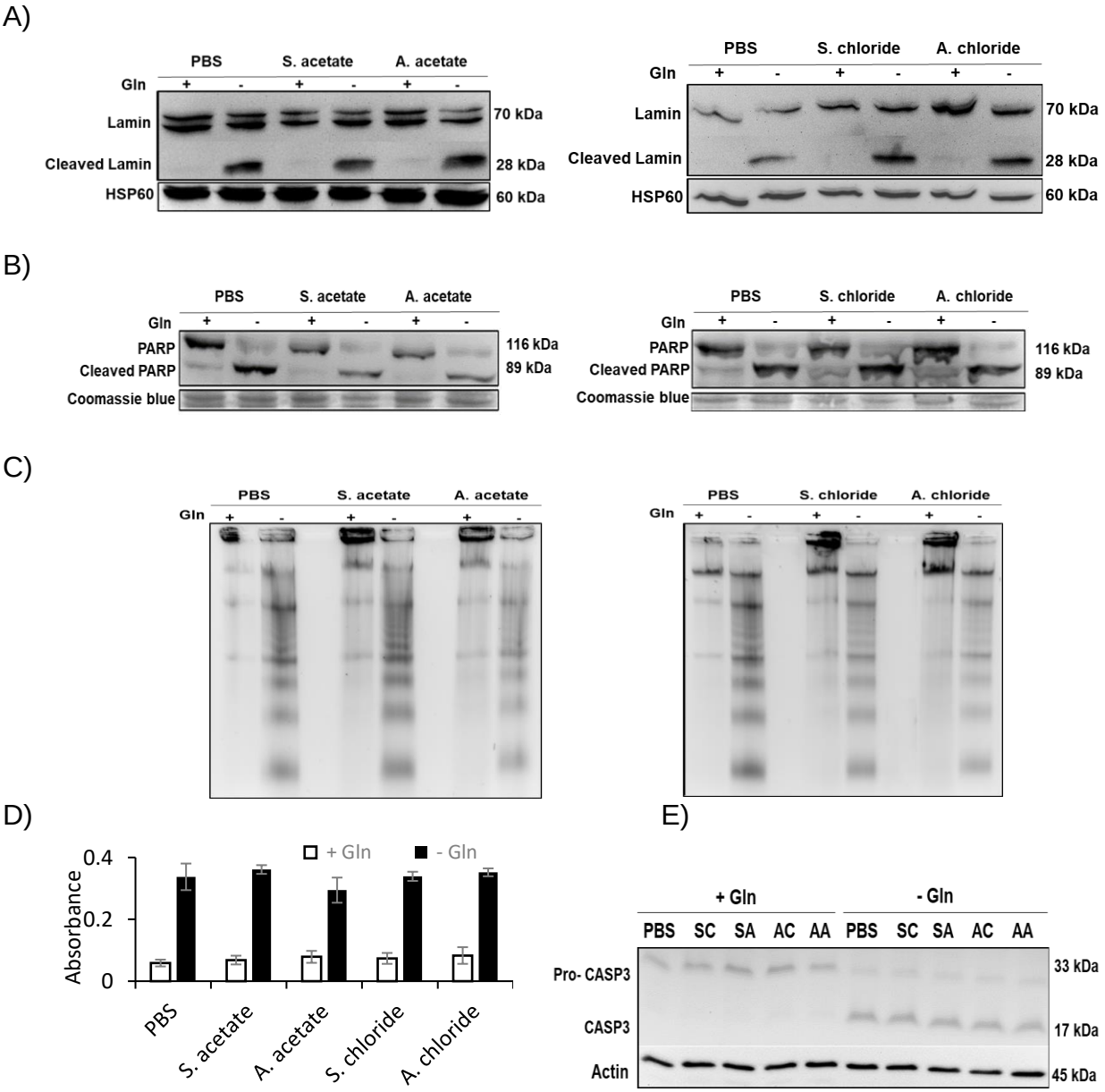


Figure 3-14 Ammonium salts do not prevent caspase-3 activation

Sp2/0 cells were deprived of glutamine for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. A) Cleavage of Lamin. B) Cleavage of PARP. C) DNA fragmentation analysis D) Caspase-3 activity assay. E) Western blot shows Caspase-3 processing. Each experiment was performed at least 3 times.SC: sodium chloride. SA: sodium acetate. AC: ammonium chloride. AA: ammonium acetate. In panel A HSP60 was used as a gel loading control, Coomassie blue was used for panel B and actin was used as a gel loading control in panel D.

3.1.5.4.1 Detection of caspase 3 activity after viable cell purification on Ficoll

The activation of apoptosis in glutamine-deprived Sp2/0 cells is a rapid and synchronized event, the majority of the cells showing apoptotic morphological features during the second hour of glutamine starvation (131). Thus, one possible reason why I could not detect a difference in caspase-3 activity in glutamine-starved Sp2/0 cells upon ammonium salt supplementation may be the presence of a significant background of apoptotic cells in the culture. To determine if this was the case, I used centrifugation on Ficoll to separate dead (denser) cells from live (lighter) cells. Moreover, I decreased the time of glutamine starvation from 3h to 2h to reduce the number of cells undergoing apoptosis. First, I tested if I can separate live from dead cells in the presence and absence of glutamine and then investigate the cleavage of caspase-3 in each fraction. As shown in Figure 3-15A, caspase-3 cleavage in the live cell fractions (with or without glutamine), was not detected. However, caspase-3 cleavage was present in the dead cell fractions confirming a successful separation of live and dead cells. Then I moved to test this approach on glutamine-starved Sp2/0 cells. There was no evidence of caspase-3 cleavage and activation in the live cells fraction whether the glutamine-starved cells had been supplemented or not with ammonium salts, PBS or the corresponding sodium salts (Figure 3-15B/C).

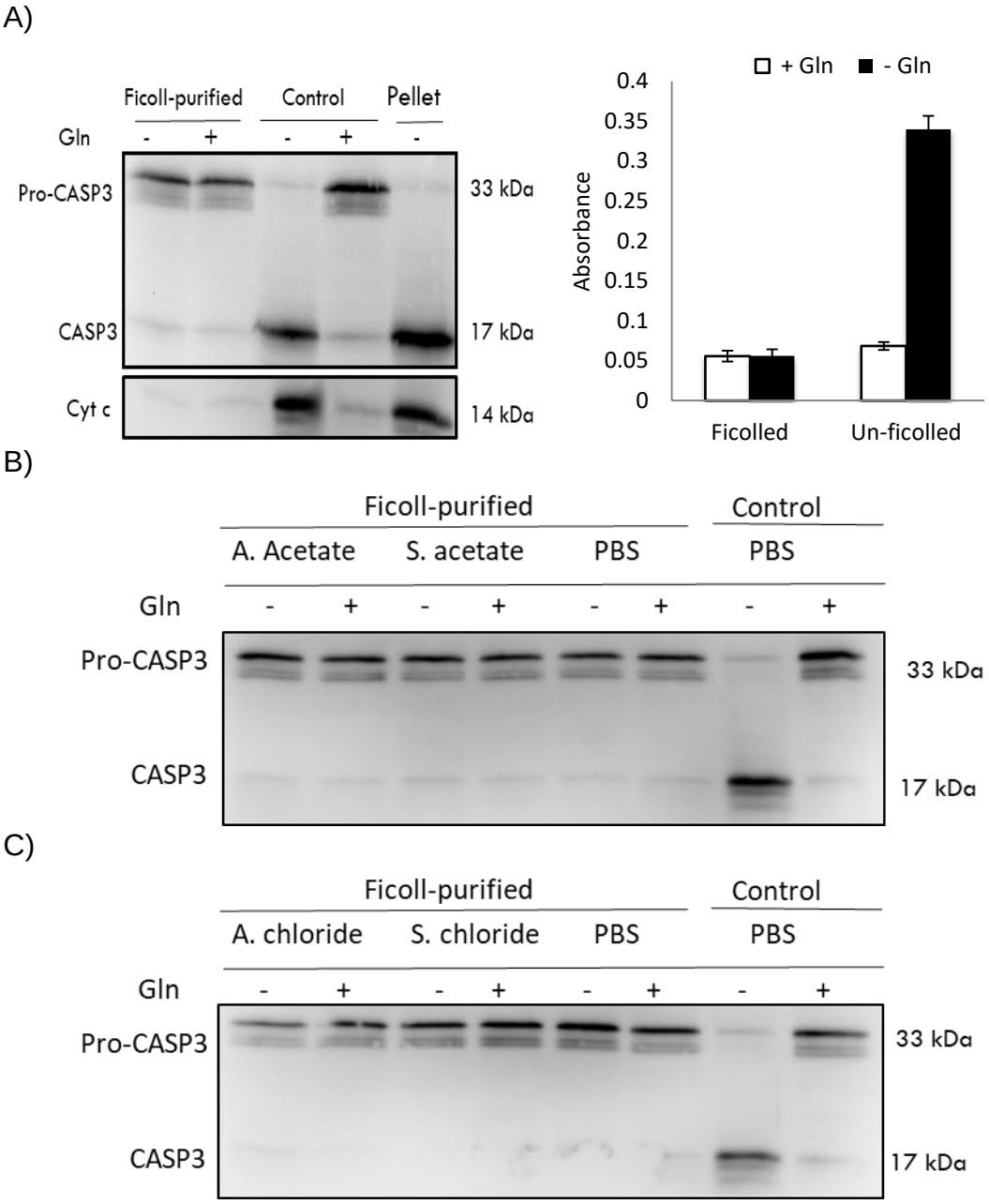


Figure 3-15. Caspase-3 cleavage and cytochrome c release are not due to the presence of dead cells in culture (Ficoll treatment)

Sp2/0 cells were deprived of glutamine (Gln) for 2 hrs. and then were separated on Ficoll gradients 2x to remove all dead cells. Equal protein concentrations were used to determine caspase-3 activity and cleavage as well as cytochrome c release by Western blot and caspase-3 colorimetric assay. A) Proof of concept of Ficoll experiment. B) Ammonium acetate. C) Ammonium chloride. Each experiment was performed at least 3 times. Ficoll purified: cells visible at the interface (live cells), control: Ficoll-untreated, pellet: Ficoll-purified (dead cells). S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

3.1.5.5 Ammonium salts don't prevent cytosolic release of cytochrome c

When Sp2/0 cells were deprived of glutamine, intrinsic apoptotic pathways are rapidly activated. This process involves the permeabilization of the mitochondrial membrane and release of cytochrome c, a component of OXPHOS machinery, from the intermembrane space to the cytosol. To examine if the pro-survival effect exerted by ammonium ions was due to its interference with this event, Sp2/0 cells were deprived of glutamine and supplemented with ammonium salts or corresponding sodium salts for 3 hours. The cells were then processed for Western blot analysis to detect the release of cytochrome c. As shown in Figure 3-16, ammonium salts did not prevent the release of cytochrome c from the mitochondria, indicating that the cytoprotective effect of ammonium ions on cell viability is not due to its interference with this event in apoptotic machinery.

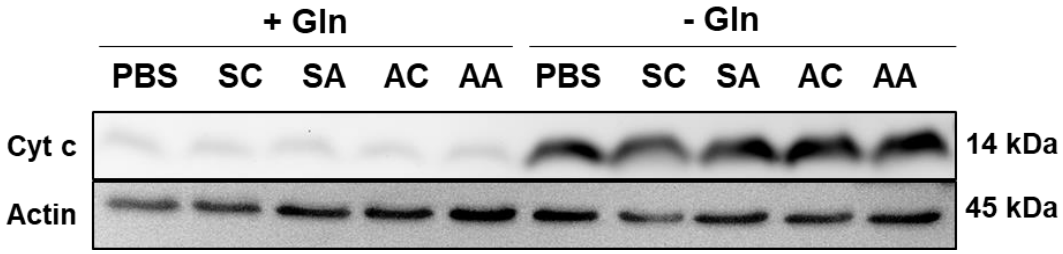


Figure 3-16 Ammonium salts do not interfere with the cytosolic release of cytochrome c

Sp2/0 cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/ sodium salt or an equivalent volume of PBS then prepared for Western blot analysis. SC: sodium chloride. SA: sodium acetate. AC: ammonium chloride. AA: ammonium acetate. Actin was used as a gel loading control.

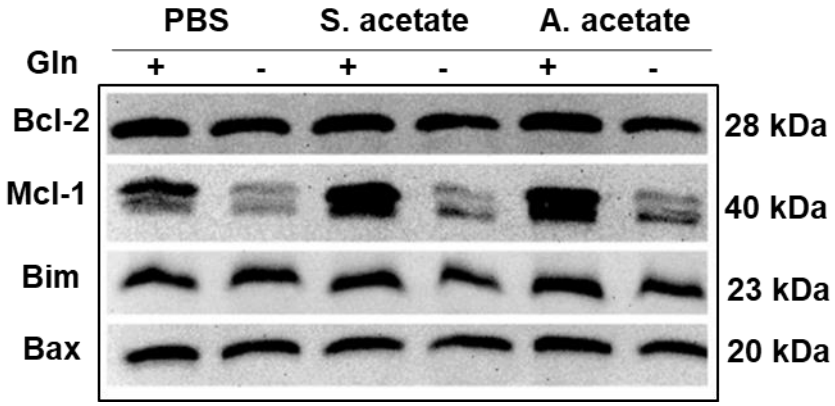
3.1.5.6 Ammonium salts don't affect BCL-2 proteins levels

Glutamine deprivation modulates the expression of some BCL-2 family members in Sp2/0 cells, namely BCL-2, MCL-1, and BAX (131,160). Therefore, I decided to investigate the effect of ammonium salts on the expression of selected BCL-2 family members. Sp2/0 cells were deprived of glutamine and supplemented with ammonium salts or corresponding sodium salts for 3 hours. The cells were then processed for Western blot analysis to detect the expression levels of a number of BCL-2 family members. Figure 3-17 shows that ammonium salts had no impact on the level of expression of the investigated BCL-2 family members, indicating that the cytoprotective effect of ammonium ions is not due to its interference with the expression of these important proteins in the apoptotic machinery.

3.1.5.7 Ammonium salts decrease apoptotic nuclei in glutamine-starved Sp2/0 cells

Having established that ammonium ions improved cell viability upon glutamine starvation without affecting the core apoptotic machinery, I turned my attention to nuclear condensation and fragmentation, two late apoptotic events which are known to occur in Sp2/0 cells deprived of glutamine (131). To achieve this, Sp2/0 cells were deprived of glutamine and supplemented with ammonium salts (or the corresponding sodium salt) for 3 hours. Nuclear morphology was then examined by Hoechst staining and fluorescence microscopy.

A)



B)

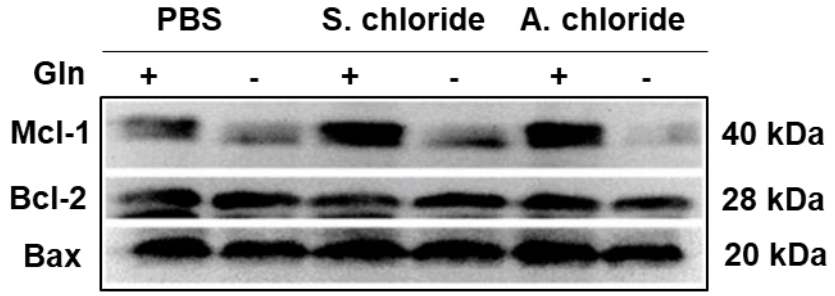
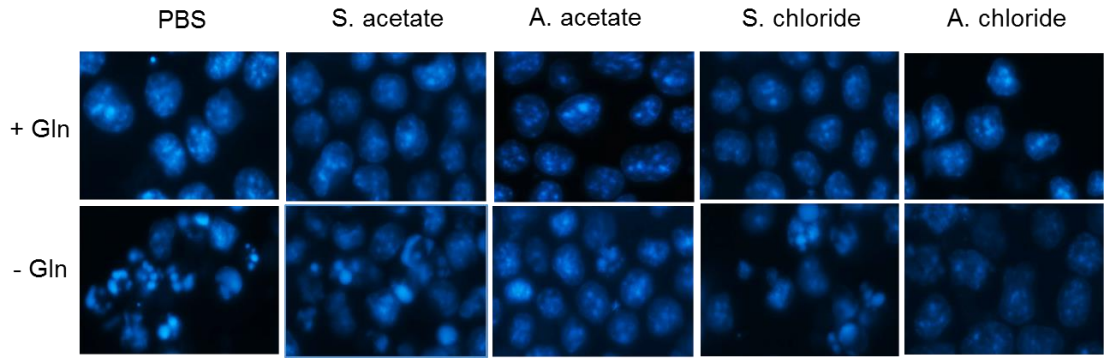


Figure 3-17 Ammonium ions do not interfere with BCL-2 proteins

Sp2/0 cells were deprived of glutamine for 3h in the presence of 5 mM ammonium/ sodium salt or an equivalent volume of PBS. A) Effect of AA on Bcl-2 proteins. B) Effect of AC on Bcl-2 proteins SC: sodium chloride. SA: sodium acetate. AC: ammonium chloride. AA: ammonium acetate. In panel A, Actin was used as a gel loading control. Figure provided by Catherine Zhou.

As shown in Figure 3-18A, the number of cells containing apoptotic nuclear condensation and fragmentation was significantly lower when cells were treated with ammonium salts compared to sodium salts or PBS controls. Upon enumeration, I found that both AA and AC led to a significant decrease in the number of cells with apoptotic nuclei compared to the controls (Figure 3-18B). All together, these experiments suggest that ammonium ions modulate the viability of glutamine-starved Sp2/0 cells in part by affecting late apoptotic events.

A)



B)

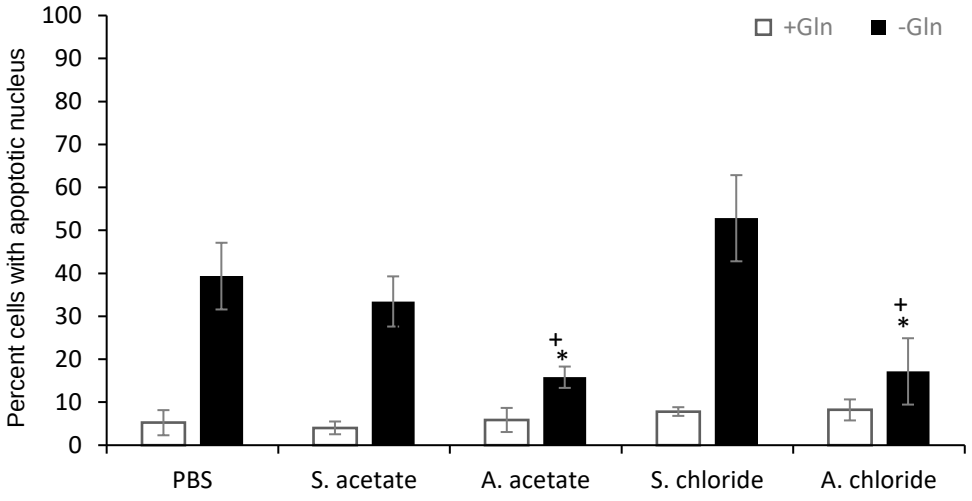


Figure 3-18 Ammonium ions lower apoptotic nuclei in glutamine-deprived Sp2/0 cells

Sp2/0 cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Hoechst 33342 was added to the cultures 30 min before the end of the experiment. The cells were then processed for fluorescence microscopy. A) Fluorescence microphotographs (magnification: 400x). B) Enumeration of cells showing apoptotic nuclear condensation/fragmentation. At least 200 nuclei were enumerated for each group. Data are the average ± SD of 3 independent experiments. *$p<0.05$ vs. PBS-supplemented control. +<0.05 vs. corresponding sodium salt-supplemented control. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

3.2 Glutamine analogs and ammonium ions

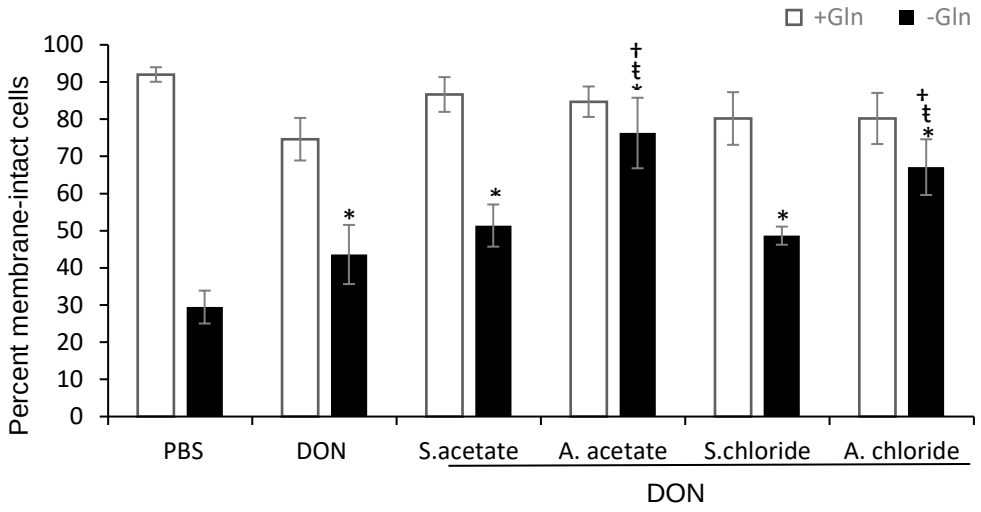
In this section, I analyzed two glutamine analogs, DON and AZA, for their effect on Sp2/0 cells. Glutamine analogs compete with glutamine and block glutamine-metabolizing enzymes by irreversibly binding to them. I wished to examine if the increased viability of Sp2/0 cells upon supplementation with ammonium ions involved the glutamine-metabolizing enzymes and see if combining glutamine analogs with ammonium ions would interfere with the latter's ability to improve the survival of glutamine-starved Sp2/0 cells.

3.2.1 Treating glutamine-deprived Sp2/0 cells with ammonium salts and a glutamine analogue significantly improves cell survival

Considering that glutamine analogs triggered Sp2/0 cells death in the presence of glutamine (Figure 3-4), I wondered whether DON or AZA would potentiate the cell death signal triggered by glutamine starvation. A dose response experiment to investigate the effect of different concentraions of DON and AZA was performed to determine the optimum concentration with reduced cell death in glutamine supplemented Sp2/0 cells (Appendix B). Therefore, Sp2/0 cells were deprived of glutamine for 3 h, in the presence of either DON or AZA. Cells to which PBS was added were used as controls. To our surprise, both glutamine analogs led to an increase in Sp2/0 cell viability upon glutamine starvation (Figure 3-19). These results prompted us to investigate whether the combination of glutamine analogs and ammonium ions could lead to a greater increase in the viability of glutamine-starved Sp2/0 cells. Sp2/0 cells were deprived of

glutamine for 3h in the presence of ammonium salts and either DON or AZA. As controls, cells supplemented with PBS or the corresponding sodium salts were also used. Interestingly, culture supplementation with the combination of AA or AC and DON led to significantly higher cell viability, to approximately 75% and 70%, respectively, compared to the PBS-treated group (Figure 3-19A). Of note, the combination of SA or SC with DON also caused a significant increase in cell viability by approximately 50% compared to 30% in PBS group. Sp2/0 cells deprived of glutamine and supplemented with either AA or AC with AZA also showed a significant increase in viability (Figure 3-19B). Interestingly, for both glutamine analogs, AC was less potent at increasing the viability of glutamine-starved Sp2/0 cells compared to AA (Figure 3-19). These results indicate that the combination of ammonium salts and glutamine analogs were able to significantly protect the glutamine-starved Sp2/0 cells against cell death.

A)



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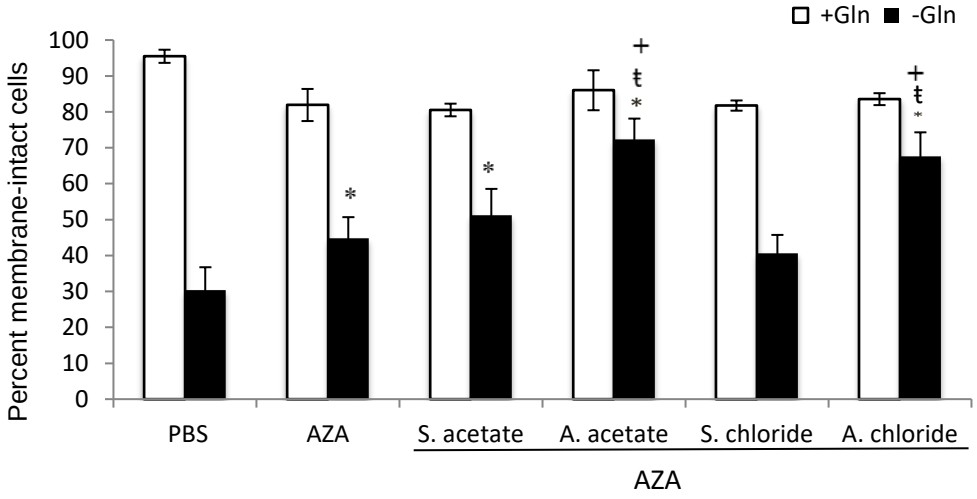
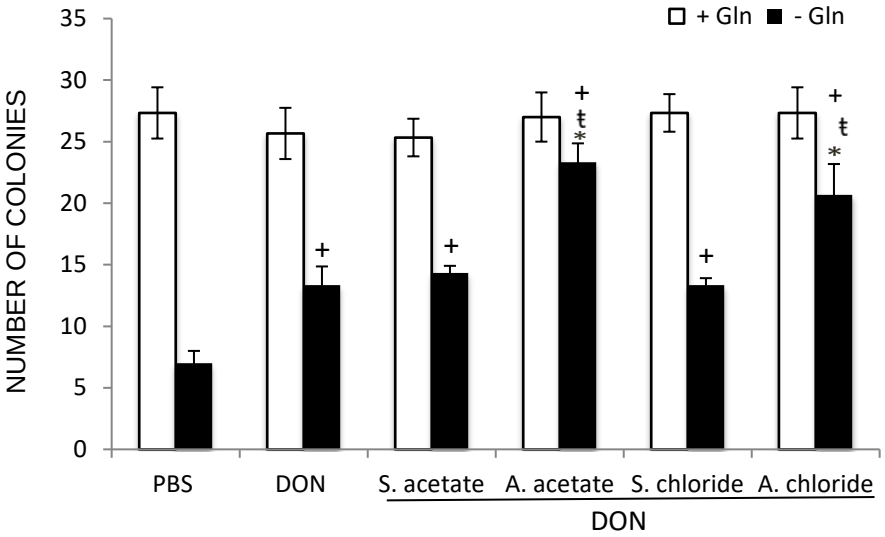


Figure 3-19 Combination of ammonium salts and DON/AZA protect glutamine-deprived Sp2/0 cells

Cells were deprived of glutamine (Gln) (black bars) for 3h in the presence of 5 mM ammonium /sodium salt or an equivalent volume of PBS. One µM of DON (Panel A) or 2µM of AZA (panel B) were added at the start of the experiment. After 3 h, cells were washed 2X with fresh IMDM then Gln (4 mM) was then added and culture was resumed for 24 h. Cell viability was determined by trypan blue exclusion. In all controls, Gln was added at the start of the experiment. Data are the average ± SD of 3 independent experiments. *p<0.01 vs. PBS. ŧp<0.05 vs. DON or AZA. $^{+}$p<0.05 vs corresponding sodium salt- supplemented control/DON/AZA. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

To confirm these results, I performed a clonogenic assay. Sp2/0 cells were deprived of glutamine for 3 h in the presence of a glutamine analog and an ammonium salt. The cells were then processed for a clonogenic assay, as described in the Materials and Methods section 2.2.4. As shown in Figure 3-20, glutamine-starved cells treated with DON or AZA alone showed an increase in colony formation compared to the control, confirming the results shown in Figure 3-19. Moreover, the co-treatment of glutamine-deprived cells with AA or AC and DON led to a significant increase in the number of colonies over the sodium salt control. Similar results were obtained when AZA was used as a glutamine analog. Importantly, the increase in colony formation was greater when the combination of glutamine analog/ ammonium salt was used than when the ammonium salts were used alone. All together, these results clearly indicate that the combination of a glutamine analog and an ammonium salt results in a much greater preservation of proliferative capacity when Sp2/0 cells are deprived of glutamine.

A)



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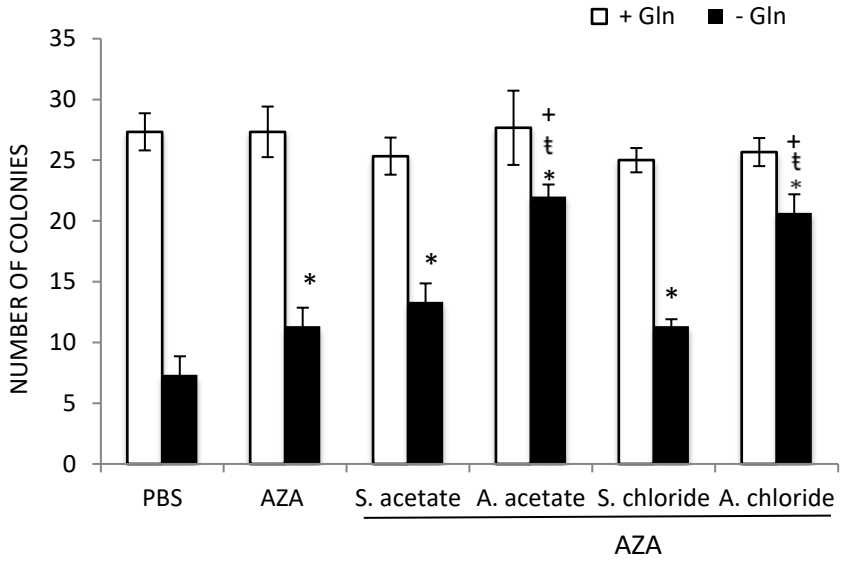


Figure 3-20 Combination of ammonium salts and DON/AZA increase proliferation of glutamine-deprived Sp2/0 cells in clonogenic assay

Cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. One µM of DON or 2µM of AZA were added at the start of the experiment. Cells were then processed for clonogenic assay. Briefly, cells were cultured at a cell density of 0.5 cells/well in 96 well plate cultured in IMDM + 4 mM Gln. After 7-10 days, colonies over ~50 cells per well were counted. Data are the average ± SD of 3 independent experiments. *p<0.01 vs. PBS. ŧp<0.05 vs. DON or AZA. +p<0.05 vs corresponding sodium salt- supplemented control/DON/AZA. A) DON. B) AZA. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

3.2.2 The combination of glutamine analogs and ammonium salts impedes the apoptotic process

3.2.2.1 Cell morphology

I next sought to determine whether the combination of glutamine analog and ammonium salt interfered with the induction of apoptosis in glutamine-starved Sp2/0 cells. When cells were examined by light microscopy, it was apparent that the use of glutamine analogs alone did not have a significant impact on morphology, with the glutamine-deprived cells showing typical signs of apoptosis (shrinkage, cell fragmentation, apoptotic body formation) (Figure 3-21). On the other hand, co-treatment of glutamine-starved Sp2/0 cells with ammonium salts and DON led to a cell morphology that resembled that of the controls supplemented with glutamine (Figure 3-21A). Similar results were obtained when glutamine deprived Sp2/0 cells were co-treated with ammonium salts and AZA (Figure 3-21B). Thus, these data suggest that the combination of a glutamine analog and ammonium salts is effective in inhibiting apoptosis.

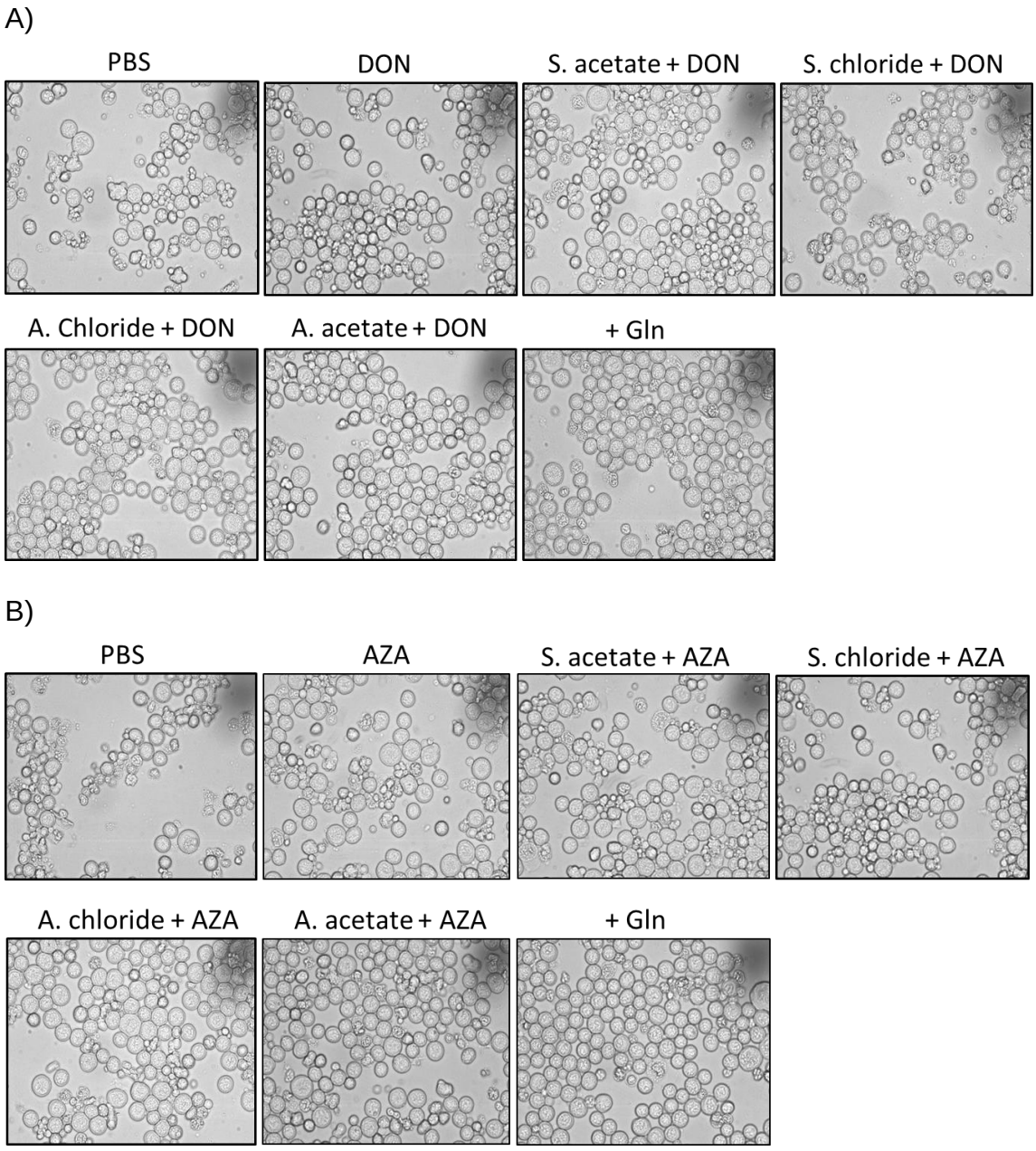


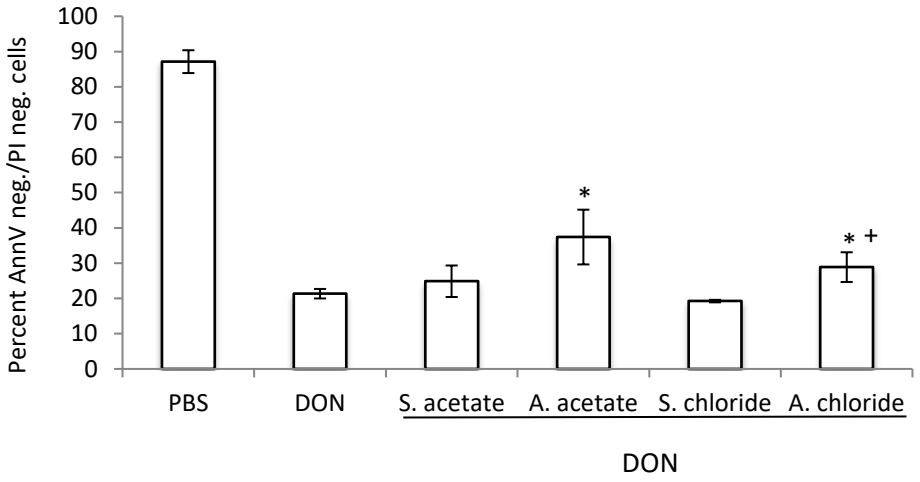
Figure 3-21 Combination of ammonium salts and DON/AZA improve cell morphology

Cells were deprived of glutamine (- Gln) for 3 h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. One µM of DON or 2µM of AZA were added at the start of the experiment Cultures in which Gln was added at the start of the experiment (+ Gln) were included as controls. A) DON. B) AZA. Each experiment was repeated at least 3 times. Magnification: 400x. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

3.2.2.2 Phosphatidylserine externalization

To confirm that the combination of a glutamine analog and ammonium salts was effective in inhibiting apoptosis in Gln-starved Sp2/0 cells, I used the AnnV-PI assay. This assay allows one to determine if phosphatidylserine externalization, a phenomenon typical of apoptotic cells, had occurred. I already showed that treatment of Sp2/0 cells with either DON or AZA in the presence of glutamine leads to approximately 80% death (Figure 3-4 and 3-5). I also showed that this effect was partially negated by the supplementation with ammonium salts (Figure 3-5). To investigate if this phenomenon involved the inhibition of apoptosis, I used the Annexin V / Propidium iodide (AnnV-PI) assay. A significant decrease in the number of viable (AnnV negative / PI negative) cells was observed when Sp2/0 cells were exposed to either DON or AZA (Figure 3-22). Interestingly, the addition of ammonium salts led to a significant increase in the number of viable cells. Thus, this confirms that ammonium salts impede the induction of apoptosis by these analogs, even in the presence of glutamine.

A)



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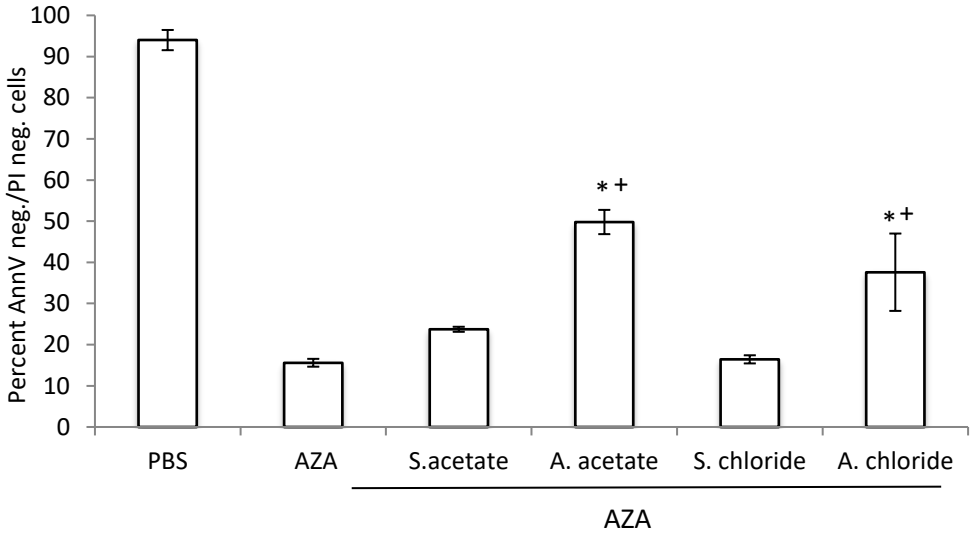

Figure 3-22 Combination of ammonium salts and DON/AZA in the presence of glutamine reduce Annexin-V externalization and PI uptake.

Cells were cultured with glutamine for 24h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. One µM of DON or 2µM of AZA were added at the start of the experiment. Cell viability was determined by flow cytometry. Data are the average ± SD of independent experiments. *p<0.05 vs. DON or AZA. +p<0.05 vs corresponding sodium salt- supplemented control. DON: 6-Diazo-5-oxo-L-norleucine, AZA: azaserine, S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

To confirm whether the combination of glutamine analog and ammonium salts inhibited apoptosis in Glutamine-starved cells, I used the AnnV-PI assay. Sp2/0 cell cultures deprived of glutamine and supplemented with DON or AZA showed a significant increase in the number of viable cells, as indicated by AnnV/PI staining. Moreover, when cell cultures were supplemented with either DON or AZA in the presence of either AA or AC, a larger increase in the number of viable cells was observed (Figure 3-23). Therefore, these results confirm that a combination of glutamine analog with ammonium did indeed interfere with apoptotic processes in glutamine-deprived Sp2/0 cells.

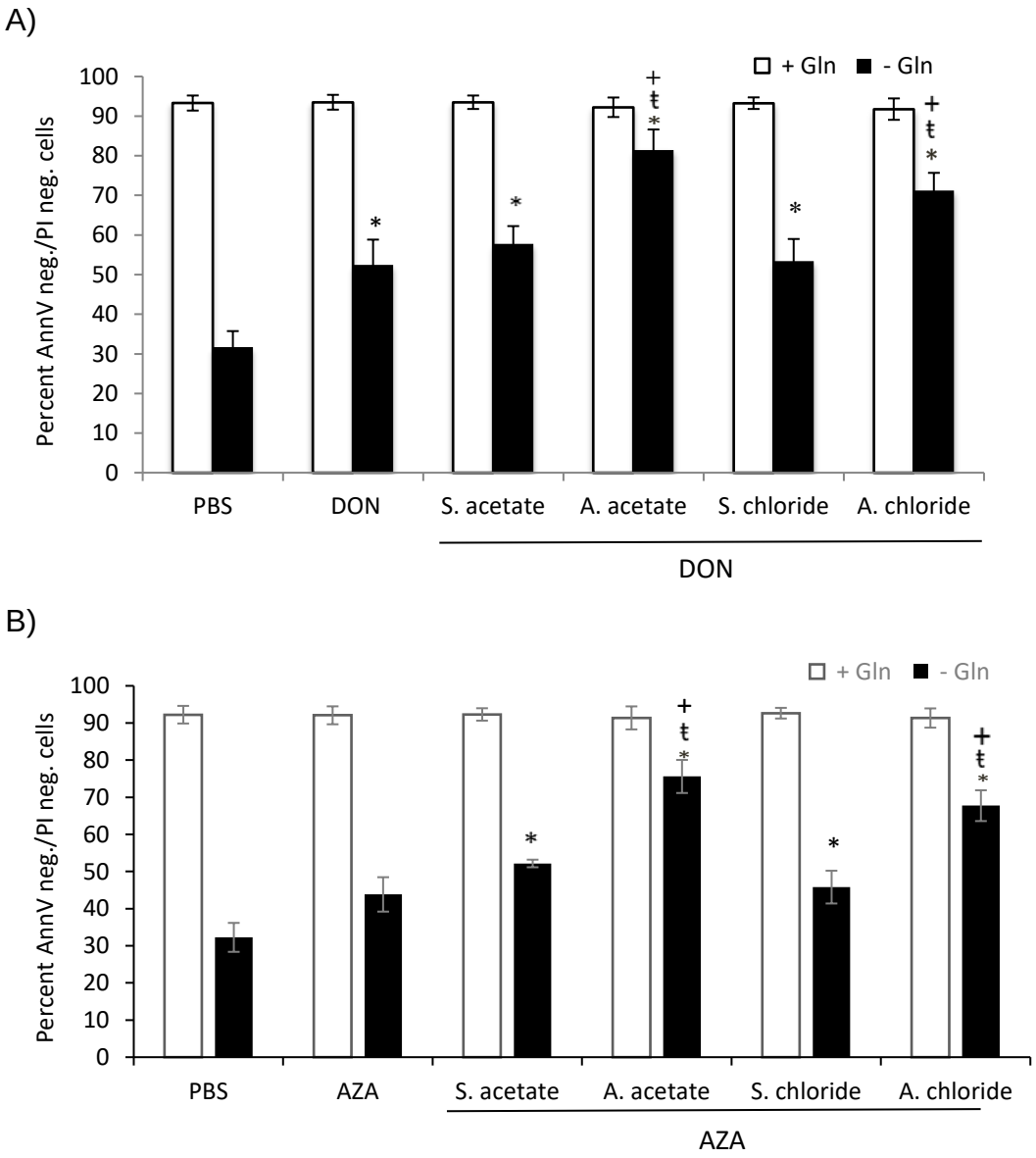

Figure 3-23 Treatment with ammonium salts in combination with DON or AZA improves cell viability.

Cells were deprived of glutamine for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. One µM of DON or 2µM of AZA were added at the start of the experiment Cells were then processed for the determination of cell viability by flow cytometry. Cultures in which Gln was added at the start of the experiment were included as controls. A) Ammonium acetate. B) Ammonium chloride. Data are the average ± SD of 3 independent experiments.*p<0.05 vs. PBS. ŧp<0.05 vs DON or AZA. +p<0.05 vs corresponding sodium salt-supplemented control. S. chloride: sodium chloride. S. acetate: sodium acetate. A. chloride: ammonium chloride. A. acetate: ammonium acetate.

3.2.2.3 Caspase-3 activation

The significant improvement in cell viability upon supplementation of ammonium ions and glutamine analogs, led us to investigate their effect on the activation of caspase-3. To achieve this, Sp2/0 cells were deprived of glutamine and supplemented with DON or AZA and with either AA, AC, PBS, or corresponding sodium salt for 3h. Then a caspase-3 colorimetric activity assay was performed. As shown in Figure 3-24A, a significant reduction in caspase-3 activation was observed when cells were co-supplemented with DON and either AA or AC when compared to PBS or corresponding sodium salts. These results were confirmed when I investigated the processing of the inactive form of caspase-3 to an active form by Western blotting (Figure 3-24B). As expected, glutamine deprivation caused the cleavage of the 32 kDa pro-caspase-3 to its active form. Importantly, supplementation with DON and either AA or AC caused a reduction in caspase-3 processing and lamin cleavage (Figure 3-24). Of note, a lower reduction in the processing of caspase-3 or lamin cleavage was observed when cells were supplemented with DON in the presence of AC than with AA. Similar results were obtained when glutamine deprived Sp2/0 cells treated with AZA and either AA or AC. The co-treatment lowered the activation of caspase-3 but not the cleavage (Figure 3-25A/B) as well as the cleavage of lamin (Figure 3-25C). Similar to my observations with DON, reduction of caspase-3 activation was more pronounced when the mixture of AZA and AA was used. Therefore, these results show that that co-supplementation of glutamine analog with ammonium salts to glutamine-deprived cells can significantly interfere with apoptosis machinery, at least in part, by limiting the activation of caspase-3.

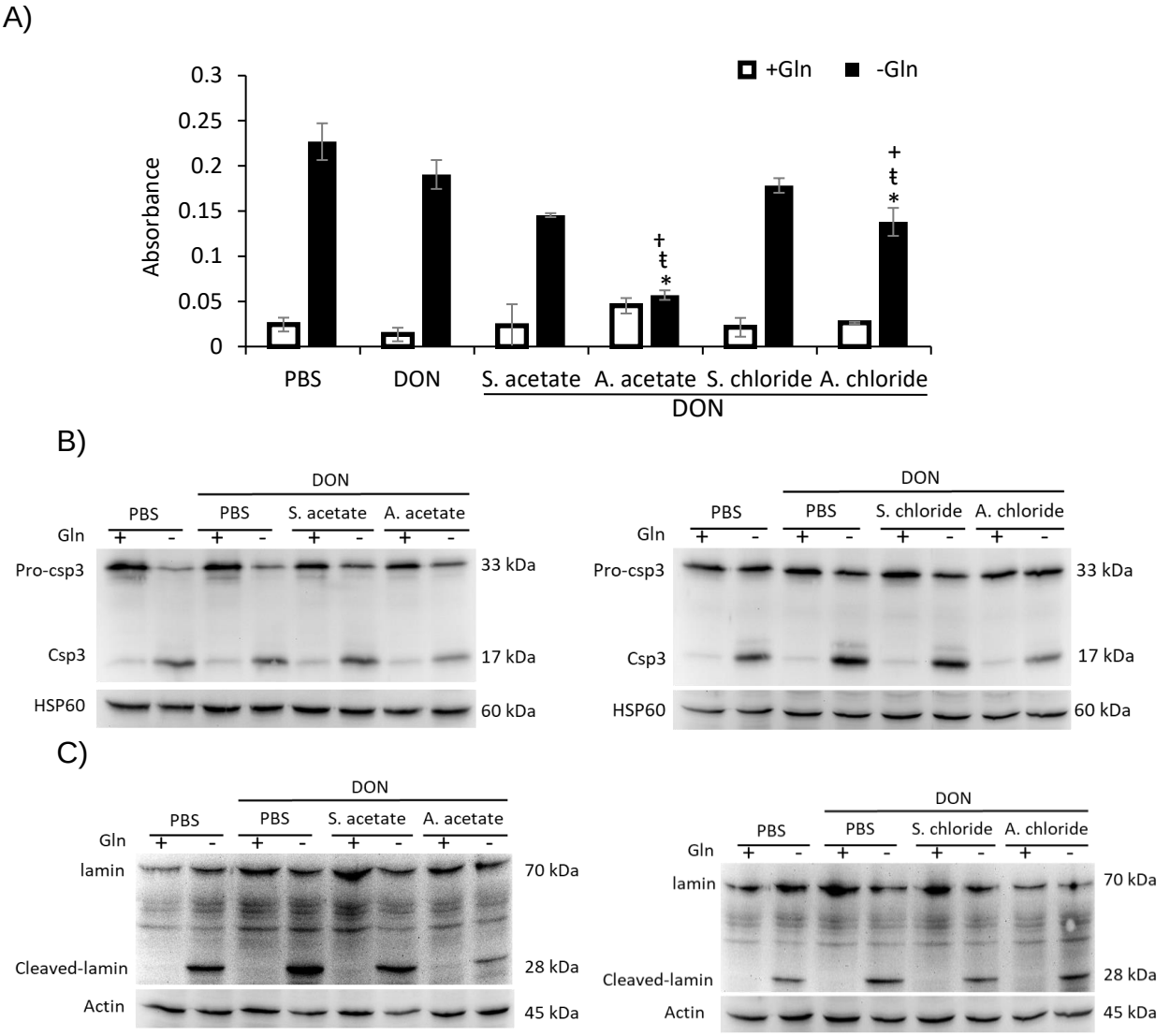

Figure 3-24 Combination of ammonium salts and DON inhibits caspase-3 or lamin cleavage

Sp2/0 cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. One µM of DON was added at the start of the experiment A) Caspase-3 activity assay. Data are the average ± SD of 3 independent experiments. *p<0.05 vs. PBS. ŧp<0.05 vs DON. +p<0.05 vs. corresponding sodium salt-supplemented control. B) Western blot shows Caspase-3 processing, full-length and the cleaved forms, respectively. C) Cleavage of Lamin. SC: sodium chloride. SA: sodium acetate. AC: ammonium chloride. AA: ammonium acetate. HSP60 and Actin were used as gel loading controls. Densitometry of Western blot bands (B and C) is presented in Appendix F.

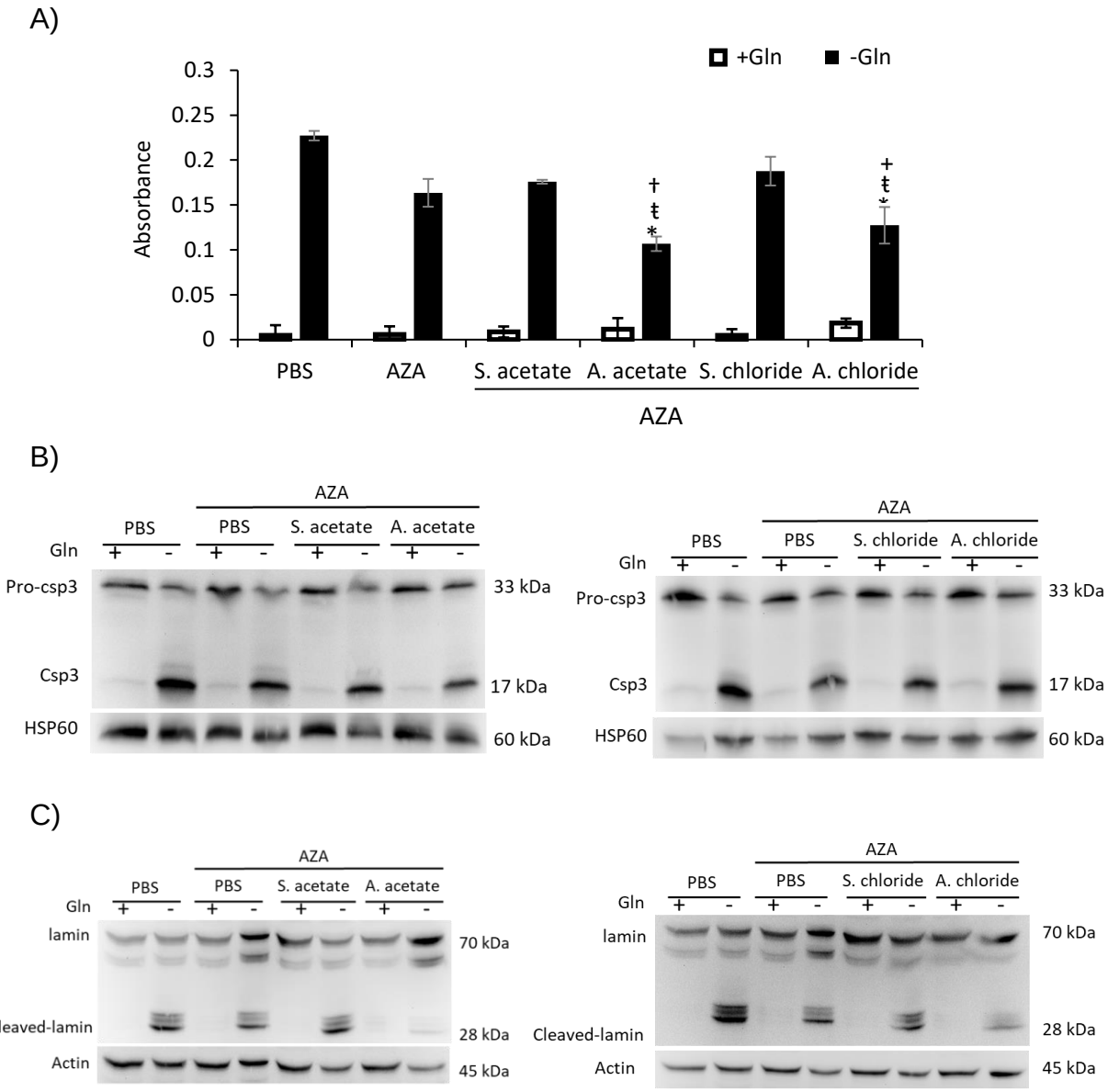

Figure 3-25 Combination of ammonium salts and AZA inhibits caspase-3 or lamin cleavage

Sp2/0 cells were deprived of glutamine (Gln) for 3h in the presence of 5 mM ammonium/sodium salt or an equivalent volume of PBS. Two µM of AZA were added at the start of the experiment A) Caspase-3 activity assay. Data are the average ± SD of 3 independent experiments. .*p<0.05 vs PBS. ŧp<0.05 AZA. +p<0.05 vs. corresponding sodium salt-supplemented control. B) Western blot shows Caspase-3 processing, full-length and the cleaved forms, respectively. C) Cleavage of Lamin. SC: sodium chloride. SA: sodium acetate. AC: ammonium chloride. AA: ammonium acetate. HSP60 and Actin were used as gel loading controls. Densitometry of Western blot bands (B and C) is presented in Appendix G.

4. DISCUSSION

The better we understand the molecular mechanisms of cancer initiation and development, the more new avenues will be available for developing new treatments for this disease and improve its prognosis and survival. The metabolic reprogramming that is specific for proliferating and cancer cells is a very attractive strategy to develop anticancer drugs (192). In that regard, the dependence of cancer cells on glutamine for their growth and proliferation is of particular interest (32,51,54,193). While the importance of glutamine for cell viability has been known for decades (194), the molecular mechanisms underlying this phenomenon are still largely unclear.

In this thesis, I investigated the effect of ammonium ions, a by-product of glutamine metabolism, on the induction of apoptosis in glutamine-deprived Sp2/0 cells. My data showed that ammonium ions significantly improved cell viability, PS externalization and nuclear condensation of glutamine-deprived Sp2/0 cells. The promotion of cell survival by ammonium ions occurred without reducing the activation of caspase-3 nor the release of cytochrome c in glutamine-starved Sp2/0 cells. The improvement of the viability of glutamine-deprived Sp2/0 cells upon exposure to ammonium ions despite the activation of caspase 3 is puzzling. However, caspase 3 activation itself does not necessarily lead to cell death. For instance, caspase 3 activation (in the absence of apoptosis) was found to be involved in the differentiation of different types of cells including muscle cells, monocytes, erythroid cells and embryonic stem cells as well as regulation of cell cycle in B cells (195-200). The mechanisms by which cells evade

apoptosis while caspase-3 is activated, are still poorly understood (196,201,202). Cells can counterbalance the progression of apoptosis upon caspase activation by upregulating the expression of antiapoptotic proteins such as IAPs and BCL-2 proteins or activate pro-survival pathways (202). It was reported that caspase-3 could be activated in healthy cells in order to circumvent mild stress through activation of anti-apoptotic AKT kinase upon partial cleavage of RasGAP by caspase 3 (196,203-205). Caspase 3 was also found to be essential for neuroprotection after preconditioning, a phenomenon which required HSP70 expression (206).

4.1 Potential mechanisms for the modulation of cell survival by ammonium ions in glutamine-deprived Sp2/0

Recently, emerging roles of ammonia in the modulation of cell viability were uncovered (207-210).

4.1.1 Heme oxygenase-1

Heme oxygenase-1 (HO-1) is an enzyme that catabolizes heme to generate iron, carbon monoxide (CO) and biliverdin. The latter is subsequently catabolized by biliverdin reductase into bilirubin. HO-1 was reported to promote the viability and function of endothelial cells (207,211,212) Researchers also showed that ammonia induces HO-1 expression (213) and recently it was reported that HO-1 promotes endothelial cell survival by regulating cellular prooxidant iron and production of carbon monoxide (CO) which are potent antiapoptotic factors (207,209,214). Endothelial cells

produce high rates of ammonia through glutamine catabolism through glutaminase activity (215). Whether HO-1 modulates the survival of Sp2/0 cells and is a target of ammonium ions in this cell line remains to be determined.

4.1.2 Contribution of autophagy

A number of studies have uncovered a possible link between glutamine metabolism and autophagy, a cell response usually associated with the promotion of survival (216). Jardon et al. recently reported that glutamine deprivation stimulates autophagy (217). In contrast, glutamoptosis is a new form of cell death characterized by the ability of glutaminolysis to inhibit autophagy via its activation of mTORC-1 (218,219). In cells undergoing glutamoptosis, conditions of nutrient limitation (i.e. with only glutamine and leucine provided as amino acids) trigger the inhibition of autophagy, resulting in the activation of caspase-8-dependent apoptosis (218,219).

Interestingly, it was recently reported that ammonium ions play several roles in regulating autophagy. Eng *et al.* reported that ammonium ions decreased TNF-α-induced cell death and that was due to an increased level of autophagy (161). Moreover, ammonium ions have been shown to stimulate mTORC2 and mTORC1 in a dose-depended manner: ammonium ions activate mTORC2 through the release of stored calcium from endoplasmic reticulum and mTORC1 through stimulation of AKT-dependent inhibition of two mTORC1 negative regulators, TSC complex and PRAS40 (220).

A link between glutaminolysis (and ammonium ions) and autophagy is interesting considering that a high rate of autophagy is detected in some types of cancer (221,222). For example, it was reported that the constitutively high level of autophagy in lung adenocarcinoma cells enables them to survive in a low-oxygen or nutrient-restricted environments. Furthermore, when autophagy was inhibited, viability of these cells was decreased (223). Interestingly, autophagy was found to be under the control of c-Myc, a decrease in c-Myc protein levels was recently correlated with a reduction in autophagy (224,225). In rapidly proliferating and cancer cells, c-Myc is known to reprogram metabolic pathways and that change leads these cells to be more dependent on glutamine. Therefore, induction of autophagy, in this case, would provide the necessary components for cell survival and proliferation. Thus, increased autophagic flux upon supplementation of ammonium ions to Sp2/0 cells cultured in glutamine-free media could promote their survival.

Several attempts were made to measure autophagy in glutamine-deprived Sp2/0 cells supplemented with ammonium ions. However, I faced several difficulties. Using Western blot analysis to detect LC3-I and LC3-II (two biochemical markers of autophagy) in Sp2/0 cells proved to be unreliable, as the signal was very weak and inconsistent. This prompted me to attempt the transfection of Sp2/0 cells with GFP-tagged proteins commonly used as markers for autophagy. While the transfection (stable or transient) of Sp2/0 cells with a GFP-expressing vector worked, transfecting the cells with a LC3-GFP fusion protein (to detect the formation of autophagosomes) was unsuccessful: the ectopic expression of LC3-GFP was toxic to Sp2/0 cells. I also

attempted to transfect Sp2/0 cells with a WIPI1-GFP fusion protein (WIPI-1 accumulates at the phagophore of cells undergoing autophagy (226-228). Here again, I was unsuccessful at expressing the fusion protein, probably due to its toxicity in Sp2/0 cells. It should be mentioned that, in previous studies performed in our laboratory, it was observed that Sp2/0 cells undergo apoptosis upon exposure to nanomolar amounts of the autophagy inhibitor bafilomycin A1 (183). The difficulty in studying autophagy in Sp2/0 cells may be an indication that this process is critical to the survival of this cell line, and that small disturbances in the autophagy process are sufficient to trigger a cell death response. More studies are required to test this possibility.

Qiang et. al., have recently reported that p38 activation is controlled by autophagy to promote cell survival during genotoxic stress (229). They showed that in cells that were sensitized to autophagy deficiency, UVB-induced apoptosis is triggered as a result of p62-mediated p38 activation. They also showed that in squamous cell carcinoma, autophagy was activated and that was in correlation with decreased activation of p38. Furthermore, they concluded that autophagy promotes cell survival by suppressing p62-mediated p38 activation (229). Studies performed in our laboratory have shown that in glutamine deprived Sp2/0 cells, the p38 kinase is activated and that this activation leads to induction of typical morphological late apoptotic features such as nuclear condensation and fragmentation as well as cell membrane blebbing (140). Moreover, while the inhibition of p38 did not prevent caspase activation in glutamine-starved Sp2/0 cells, it reduced the extent of nuclear condensation/fragmentation and cell membrane blebbing. Our research group also showed that the activation of p38 in glutamine-

deprived Sp2/0 cells was in response to oxidative stress. Therefore, the lower nuclear condensation and cell membrane blebbing observed upon ammonium ions treatment of glutamine-starved Sp2/0 cells might be due to increased levels of ammonia-derived autophagy and hence lower levels of p38 activation. The possible involvement of p38 in the pro-survival response triggered by ammonium ions could be tested through the use of chemical inhibitors (e.g. SB 203580) or the transfection of Sp2/0 cells with dominant-negative versions of the p38 kinase (140).

The potential contribution of autophagy to the modulation of the survival of glutamine-starved Sp2/0 cells could be determined using alternative approaches. For instance, autophagy inhibitors such bafilomycin A1 or 3-methyl adenine could be used. As stated earlier, care should be taken when using these chemicals, as they both quite toxic to Sp2/0 cells (183). Alternatively, altering key components of the autophagic pathways (e.g. beclin-1) through RNA interference or ectopic expression approaches could provide us with useful information as to the contribution of this phenomenon to the effect of ammonium ions on Sp2/0 cells. Using flow cytometry as a method of measuring the levels of autophagy is also very promising to be useful in Sp2/0 cells (230). For example, flow cytometry can be used to measure different aspects of autophagy such as LC3 (autophagosome) accumulation, its upregulation throughout cell cycle, and co-localization of autophagosomes and lysosomes (231,232).

4.1.3 GADD-153

The stress-related transcription factor GADD-153 was reported to be up-regulated under hyperammonemic conditions (233). Moreover, GADD-153 levels were shown to be increased following the inhibition of autophagy (234). Interestingly, this transcription factor was shown to be up-regulated in glutamine-starved Sp2/0 cells (235). It was reported that upregulation of GADD-153 leads to increased expression of mitochondrial chaperone proteins which provide the mitochondria with the capability to cope with the accumulation of unfolded proteins. Therefore, it would be interesting to measure the expression levels of GADD-153 in glutamine-deprived Sp2/0 cells supplemented with ammonium ions or knockdown GADD-153 gene by small-interfering RNA and see if the protective effect of ammonium ions still occurs.

4.1.4 Contribution of acetate and α–ketoglutarate to the effect of ammonium ions

While both ammonium acetate and ammonium chloride increased cell viability, I noticed that ammonium acetate exerted a greater level of protection compared to ammonium chloride. Therefore, I asked if this increase in viability due to carbon source provided by the presence of acetate. This was investigated by treating glutamine-deprived Sp2/0 cells with methyl pyruvate (MPyr), as a replacement for acetate, in combination with ammonium chloride. The addition of MPyr did not improve in the effect of ammonium chloride on cell viability. Similar results were obtained when the amino acid glutamate was used along with ammonium chloride (Figure 3-7).

The role of acetate in promoting cell survival is not clear but it may be due to its conversion into acetyl-CoA via acetyl-CoA synthetase (ACSS2) (236,237) Acetyl-CoA can then be metabolized in the TCA cycle or used by the cell for biosynthesis of lipids. Acetyl-CoA can also act as a substrate for the synthesis of UDP-GlcNAc which can, in turn, promote cell viability (238). Therefore, in rapidly proliferating cells such as Sp2/0 cells, it is possible that acetate has a protective role in the absence of glutamine. Another explanation of the weaker effect of ammonium chloride in comparison to ammonium acetate is that ammonium chloride has been shown to induce a transitory increase in mitochondrial pH which in turn leads to an increase in production of ROS (239-243). Measuring intracellular level of ammonia or use of ROS scavengers might shed some light on this possibility.

Supplementation of α-ketoglutarate, another metabolite derived from glutaminolysis, also led to a significant increase in cell viability when added to glutamine-deprived Sp2/0 cells. This suggests that glutamine protection against cell death may be due to other metabolites of glutamine besides ammonia. This can be attributed to the effect of α-ketoglutarate on mTORC1 signaling pathway, which is responsive to nutrient starvation and links cell metabolism to autophagy. This, in turn, has been shown to influence cell survival (152,244,245). Ammonium ions can also activate mTORC1 pathway, which is usually inhibited in cancer cells due to nutrient stress (216). mTORC1 activation promotes α-ketoglutarate production through activation of glutamate dehydrogenase (152) and hence production of ammonia and stimulation of autophagy. Moreover, as α-ketoglutarate is known to activate the mTOR pathway, the result of this

interplay would be a feedback loop for constitutive stimulation of autophagy (246). The role of mTOR in Sp2/0 cells could be examined by inhibiting mTOR in the presence of ammonium ions or α-ketoglutarate. However, it was reported that ammonium ions promote autophagy independently of ULK1/ULK2 kinases, two protein kinases involved in autophagosome formation and subject to modulation by mTOR (247). Therefore, combined treatment with ammonium ions and rapamycin, an mTOR-dependent autophagy stimulator, could further elucidate the effect of ammonium ions on cell viability in glutamine-starved Sp2/0 cells. Nevertheless, no additive effect on cell viability was observed when glutamine-starved cells were treated with a combination of α-ketoglutarate and ammonium ions (Figure 3-8). This finding is in contrast to the results reported by Eng *et al.,* (161) where a synergetic effect of ammonium ions and α-ketoglutarate was observed. This is possibly the result of the fact that I had to use lower amounts of dimethyl-α-ketoglutarate in our experiments, as this chemical proved toxic to Sp2/0 cells (results not shown).

Finally, another way ammonia can contribute to cell survival is by its effect on glutathione levels. It was reported that ammonia increases the antioxidant, glutathione levels to 80% in cerebral cortical microdialysates and was also able to increase glutathione synthesis in cerebral cortical astrocytes and a C6 glioma cell line (248). It was also reported by others that supplementation of ammonia to hybridoma cells increases the activity of glutamate dehydrogenase leading to glutamine synthesis from ammonia and α-ketoglutarate (187). However, improvement in cell viability upon treatment with ammonium ions was not attributed to an increase in glutamine production

by glutamine synthetase (GS), since treating glutamine-deprived Sp2/0 cells with methionine sulfoximine did not prevent ammonium ions from improving cell viability on cell viability (Figure 3-9).

4.2 Effect of ammonium ions on intrinsic apoptotic pathway

After confirming the increased cell viability induced by ammonia treatment on glutamine-starved Sp2/0 cells, I started to study the effect of ammonia on key components of the intrinsic apoptotic pathway. Ammonium ions supplementation did reduce PS externalization, the number of cell in the sub-G1 phase in cell cycle and nuclear condensation/fragmentation in glutamine-starved Sp2/0 cells (Figures 3-9, 3-10 and 3-15). This indicates that ammonium ions interfered with the progress of the apoptotic process.

Ammonia has been shown to affect the size of the nucleus in a caspase-independent manner and increases the activity of phospholipase A2 (PLA2), an enzyme involved in nuclear shrinkage (249-251). This might explain the effect of ammonium ions on nuclear condensation and the null effect on the core components of apoptosis. It would be interesting to know if PLA2 is at play in glutamine-deprived/ammonium ion supplemented Sp2/0. This can be confirmed by using selective intracellular PLA2 activity inhibitors such as methyl arachidonyl fluorophosphonate (MAFP), bromoenol lactone (BEL), and arachidonyltrifluoromethyl ketone ($AACOCF_3$) (249) and examine whether nuclear shrinkage still occurs.

Ammonium ions were also shown by others to increase the intracellular pool of UDP-GlcNAc, involved in protein O-glycosylation, a post-translational modification known to inhibit cell death (238,252). O-GlcNAc is involved in the modification of PFK1, an enzyme involved in glycolysis, leading to increased rates of glucose entry into the pentose phosphate pathway, instead of the TCA cycle, to promote growth and survival in rapidly proliferating cells such as cancer (253,254) O-GlcNAc is involved in autophagy through the regulation of lysosomal protein degradation and this regulation depends on the conditions of nutrients availability (255). O-GlcNAc also activates AMPK and the latter is involved in cell survival against stress (256). O-GlcNAc also promotes the expression levels of HIF-1α and attenuate the activation of unfolded protein response to ER stress (257). It was also reported that UDP-GlcNAc protein is necessary for proliferation and adhesiveness of mouse embryonic fibroblasts (MEFs) deficient for EMeg32 which can be recovered by nutritional restoration of intracellular UDP-GlcNAc levels (258). In addition, different proteins in the cell are also targets for O-GlcNAcylation such as nuclear pore proteins, transcription factors, as well as chromatin-modifying enzymes (253,259,260). Therefore, detection of intracellular levels of UDP-GlcNAc in glutamine-starved/ammonium ion supplemented Sp2/0 cells would provide a good indication if UDP-GlcNAc has a role in the observed increased cell viability.

Our data showed that supplementation with ammonium salts had no impact on the core intrinsic apoptotic events such as cytochrome c release, BCL-2 family protein levels, caspase-3 activation, PARP, and Lamin A/C cleavage. The apparent lack of effect of ammonium ions on intrinsic apoptotic pathways in glutamine-starved Sp2/0 cells may be

due to the fact that ammonia was reported by others to actually induce intrinsic apoptosis via blocking complexes I-IV of the mitochondrial respiratory chain, leading to depolarization of mitochondrial membrane and hence the release of cytochrome c to the cytosol (261-263). It is difficult, however, to reconcile these events with the long-term protection afforded by ammonium ions in glutamine-starved Sp2/0 cells, and the fact that ammonium ions, on their own, did not trigger apoptosis in glutamine-supplemented Sp2/0 cells.

4.3 Ammonium ions and glutamine analogs

Next, I reported on the effect of glutamine analogs on the viability of Sp2/0 cells. Treating Sp2/0 cells with either DON or AZA was highly toxic to the cells, even in the presence of glutamine. This indicates that interfering with glutamine catabolism is sufficient to trigger Sp2/0 cell death. Treatment of Sp2/0 cells with either DON or AZA in the presence of glutamine in combination with either ammonium acetate or ammonium chloride afforded some protection from apoptosis compared to control groups (Figure 3-5).

Surprisingly, the viability of glutamine-deprived Sp2/0 cells treated with a glutamine analog and an ammonium salt was considerably improved (Figure 3-19). These results were supported by several complementary experiments: clonogenic assays, flow cytometry analysis, examination of cell morphology, as well as biochemical assays (caspase activity, Western blot analysis). However, the reasons behind the lower cleavage and activation of caspase-3 proteins are not entirely clear.

Although I only showed the effect of combined treatment of glutamine-starved Sp2/0 cells with ammonium ions and glutamine analogs on caspase-3, it would be interesting to investigate the effect of ammonium ions on other core apoptotic machinery such as the cytosolic release of cytochrome c, the modulation of BCL2 family proteins, and the requirement for other caspases. Detection of cytochrome c release through Western blotting upon supplementation of ammonium ions to glutamine-starved Sp2/0 cells as well as the examination of the levels of BAX and BAK in the cytoplasm or their activation and translocation to the mitochondria would show that ammonium ions and glutamine analogs work upstream of the permeabilization of the outer mitochondrial membrane. If cytochrome c is still released, however, that means ammonium ions and glutamine analog might interfere with the processes responsible for the activation of caspase-3.

Both ammonium ions and DON have been shown to affect the synthesis of UDP-GlcNAc and O-GlcNAc, the levels of which are linked to apoptosis (78). Glutamine analogs, DON and AZA, decrease O-GlcNAc levels by blocking glutamine-fructose-6-phosphate transaminase (GFAT), the rate-limiting enzyme of the hexosamine biosynthetic pathway (HBP) which uses glutamine as an amino group donor (264). However, ammonium ions stimulate the O-GlcNAc-modification of proteins. As both, glutamine analog and ammonium ions affect the levels of O-GlcNAc, suggests an interplay between glutamine analogs and ammonium ions via protein modification by O-GlcNAc to promote survival of glutamine-starved Sp2/0 cells. This can be confirmed by using RNA interference against O-GlcNAc or by using pharmacological inhibitors such

as O-(2-acetamido-2-deoxy-D-glucopyrano-sylidene)amino-N-phenylcarbamate (PUGNAc) (265) along with detection of protein modification by O-GlcNAc by Western blotting using anti-O-GlcNAc antibodies during ammonium ions and DON or AZA treatment.

Apoptosis is a process of events that requires energy. Amongst these events are blebbing, the formation of apoptotic bodies and chromatin condensation. Ammonia could provide a link between energy metabolism and apoptosis where the progress of these events upon glutamine starvation is compromised. For example, ammonia can be used as a nitrogen donor instead of glutamine for the conversion of D-fructose-6P (Fru6P) into D-glucosamine-6P, a reaction of hexosamine metabolism, catalyzed by Glucosamine-6P synthase (GlmS) (266). The end product of this pathway is UDP-N-acetyl glucosamine. In addition, DON has been shown to bind to the inactive glutaminase active site, leading to conformational change exposing the ammonia channel activation of the enzyme (266-268). This might, in part, promote the production of UDP-N-acetyl-glucosamine and would explain the significant improvement of cell viability when a combination of ammonium ions and DON was used (Figure 3-19).

In conclusion, our data show that ammonia ions partially protected glutamine-starved Sp2/0 cells from apoptosis. The fact that the combined treatment of ammonium ions with a glutamine analog improved cell viability to the level of glutamine-fed cells, a phenomenon accompanied by the inhibition of caspase activation, was unexpected and is very interesting. The development of more specific glutaminase (GLS) inhibitors such

as BPTES and 968, which were shown to halt glutaminolysis, has recently triggered a renewed interest in the use of glutamine analogs for cancer therapy (34,54,61,269-271). Further studies are required to investigate the effect of ammonium ions supplementation on glutamine-deprived cells.

4.4 Future work

I have established that the molecular mechanism underlying the protective effect of ammonium ions against glutamine-starvation-induced apoptosis is influenced by the presence of a glutamine analog. Whether or not the intrinsic apoptosis pathway is modulated by the combination of ammonium salts and glutamine analog is an important question that remains to be answered. It would be interesting to see whether cytochrome c was released from the mitochondria when Sp2/0 cells were co-treated with glutamine analog and ammonium ions. Whether BCL-2 proteins (and in particular MCL-1) are modulated (protein levels, protein-protein interactions, intracellular localization, post-translational modification) by ammonium ions and glutamine analogs should also be investigated. Additionally, whether autophagy is at play is not entirely clear. It has been established that autophagy and apoptosis are interconnected at different levels (272). For example, mTOR is involved in both processes (273,274), and caspase-9 and caspase-8 were found to mediate the cleavage of Beclin-1, a tumor suppressor and autophagy regulator, leading to inhibition of apoptosis (275-278). Although it was reported that ammonia stimulates autophagy independently of mTOR (161), it was recently reported that ammonia stimulates autophagy by inhibiting mTOR

via dopamine receptor D3 (DRD3) (279). It would be interesting to see if either pathway occurs in our cell line.

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6. Appendix

Appendix A Solutions, buffers and their compositions.

Solution/buffer	Composition
15x TBE buffer (1 L)	154 g Tris base 527.5 g Boric acid 420 ml 0.5 M EDTA pH 8.0
200 mM Glutamine (10ml in PBS)	0.2922 g Glutamine Adjust pH to 7.2-7.4 Filtered and stored at 4^{o} C
10X PBS (1 L)	800 ml ultrapure water 80 g NaCl 2 g KCl 14.4 g Na_2HPO_4 2.4 g KH_2PO_4 Adjust pH to 7.4 with HCl.
1X PBS (1 L)	Adjust pH to 7.4 with HCl. Bring volume to 1 liter100 ml 10x PBS 900 ml ddH_2O
10 x PBS (30 ml) (Cytosolic fraction Isolation)	0.041 g NaH_2PO_4 0.341 g Na_2HPO_4
Solution A (Cytosolic fraction Isolation) (1 ml)	100 µl of 10 x Digitonin solution 900 µl of Solution B
10X Digitonin (1 ml)	2.5 mg Digitonin 1 ml Solution B
Solution B (30 ml) (Cytosolic protein isolation)	3 ml 10x phosphate butter 0.168 g KCl (final concentration: 75 mM) 9.23 g sucrose
5 M Ammonium Acetate (10 ml in PBS)	3.85 g Ammonium Acetate Adjust pH to 7.4 with HCl
5 M Ammonium chloride (10 ml in PBS)	2.67 g Ammonium chloride Adjust pH to 7.4 with HCl
1 M Sodium Chloride (10 ml in PBS)	0.584 g Sodium chloride Adjust pH to 7.4 with HCl
1 M Sodium Acetate (10 ml in PBS)	1.360 g Sodium Acetate Adjust pH to 7.4 with HCl
1M Dimethyl 2-oxoglutarate (1 ml)	15 µl Dimethyl 2-oxoglutarate (9.6 M) 1985 µl absolute Ethanol

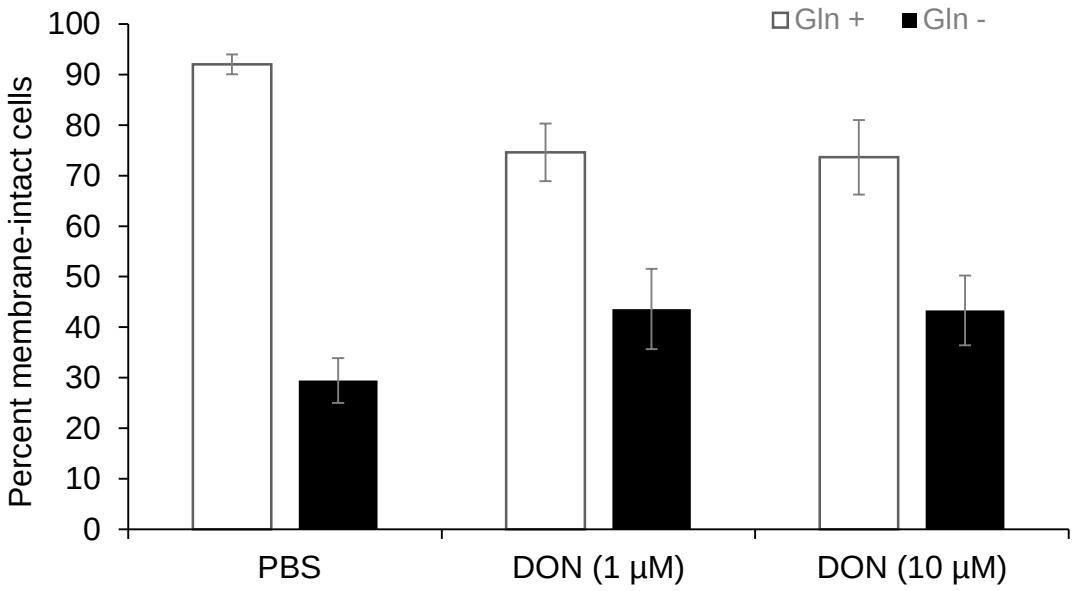
Solution/buffer	Composition
10 mM Methyl pyruvate (1 ml)	100 µl of 100 mM Methyl Pyruvate 900 µl PBS
5 M Methylamine hydrochloride (10 ml in PBS)	3.376 g Methylamine hydrochloride
200 mM Methionine sulfoximine (1 ml in PBS)	36.046 mg Methionine sulfoximine
200 mM glutamate (10 ml n PBS)	0.29426 g Glutamate
1 mM AZA (1 ml in PBS)	3.45 µl AZA
1mM DON (1 ml in PBS)	20 µl DON (50 mM)
Annexin V Binding Buffer (100 ml)	10 mM Hepes (238.3 mg) aAdjust to pH 7.4 140 mM NaCl (818.1 mg) 2.5 mM $CaCl_2$ (36.75 mg)
DNA fragmentation lysis buffer (10 ml)	***0.1*** ml of 1 M Tris-HCl, pH 8.0 0.02 ml of 0.5 M EDTA, pH 8.0 50 mg of N-lauroyl sarcosine 1.0 ml of 2 mg/ml RNase A stock 2.5 mg of Proteinase K
Loading buffer(DNA)	040% sucrose 0.08% Bromophenol Blue
SDS-PAGE	
30% Acrylamide Stock solution: (50 ml)	29.2 % Acrylamide 0.8 % Bisacrylamide
4X Tris/SDS pH 8.8 (100ml)	18.16 g Tris HCl 2ml of 20% SDS pH to 8.8
4X Tris/SDS pH 6.8 (50 ml)	T9.0885 g Tris-HCl 40 ml H_2O pH 6.8 0.2 g SDS
5X electrophoresis buffer (1L)	15.1 g Tris base 72 g Glycine 5 g SDS
Coomassie blue staining solution (250 ml)	50 %methanol 0.05 % Coomassie Brilliant Blue; 10 % Acetic Acid 40 % H_2O
De-staining Solution	50 % Methanol 10 % Acetic Acid 40 % H_2O

Solution/buffer	Composition
6X Sample Buffer (10 ml)	7 ml 4X Tris/SDS pH 6.8 3.8 g Glycerol 1 g SDS 0.6 ml 2-mercaptoethanol 2 mg Bromophenol blue
Urea Lysis Buffer (10 ml)	1 g Glycerol 1 ml of H2O and vortex 3.606 g of Urea 625 µl 1M Tris-HC1 pH 6.8 l ml H2O 500 µl β-mercaptoethanol 1 ml 20% SDS 100 µl Bromophenol Blue 100 µl PMSF (10 mg/ml in Me0H) 500 µl 1M NaF 400 µl 50 mM Orthovanadate
10% APS (10 ml)	1g Ammonium persulfate
1x TTBS (1 L)	100 ml 10x TBS 900 ml ddH_2O
Blocking buffer	1x TTBS 5% BSA
SDS-PAGE running buffer	25 mM Tris-HCl pH 8.3 190 mM Glycine 0.1% SDS
Ponceau-S staining solution (1 L)	1g Ponceau S 50 ml Acetic acid Make up to 1L with ddH_2O
Transfer buffer (800 ml, /o methanol)	500 ml ddH_2O 5.82 g Tris 2.93 Glycine 0.373 g SDS
2X RIPA (9 ml)	6 ml 3X PBS 180 µl IGEPAL 0.09 g Sodium Deoxycholate 90 µl SDS (from 20% stock solutions) Complete to 9ml with H_2O
1X RIPA (1 ml)	500 µl 2X RIPA 100 µl Proteinase Inhibitor Cocktail 50 µl NaF 1M 4 µl Orthovanadate 50 mM 10 µl PMSF (10mg/ml) 336 µl H_2O

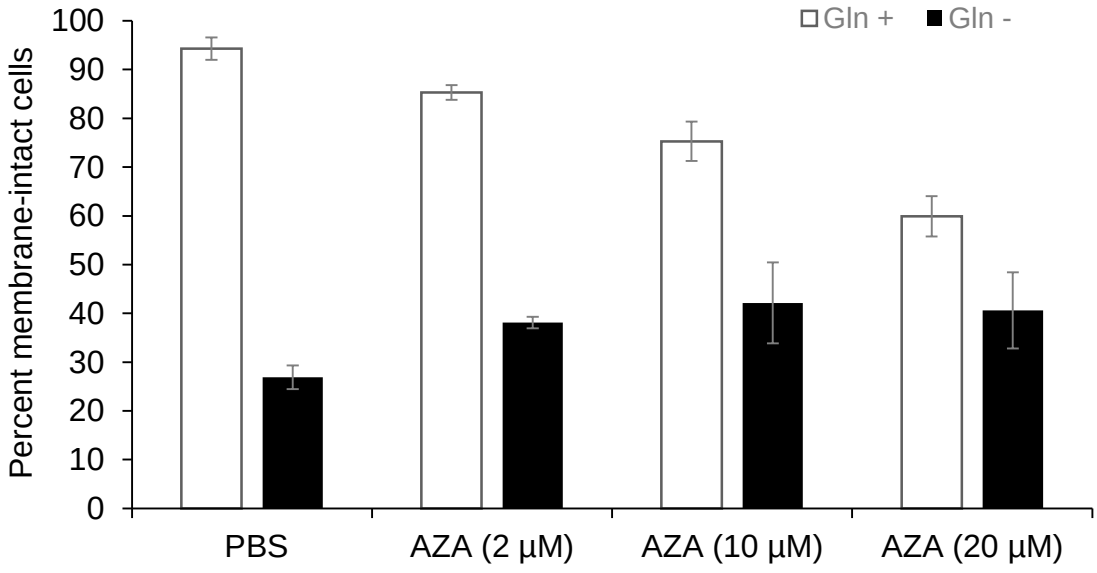
Solution/buffer	Composition
Tris-Tricine SDS	
Tris·Cl/SDS, pH 8.45 (50 ml)	18.2 g Tris base pH to 8.45 with HCl 30 ml H_2O .15 g SDS30 ml
4X Tris-Cl/SDS pH 6.8 (100 ml)	6.05 g Tris base in 40 ml dd-water Adjust pH to 6.8 with 1 N HCl. Bring volume to 100 ml with dd-water. Add 0.4 g SDS
2X Tricine Sample Buffer (10ml)	2 ml 4X Tris·HCl/SDS, pH 6.8 3 g Glycerol 0.8 g SDS 0.31 g DTT 2 mg Coomassie Blue
5x Anode Buffer pH 8.9 (1 L)	121.1 g Tris base 500 ml H_2O Add H_2O to 1 liter
1x Cathode Buffer (1 L)	12.11 g Tris 17.92 g Tricine 1 g SDS Add H_2O to 1 liter
10X TBS (1L)	24.23 g Trizma HCl 80.06 g NaCl Mix in 800 ml ultra pure water. pH to 7.6 with HCl. Top up to 1 L.

Appendix B Dose response of different concentrations of DON and AZA

A)



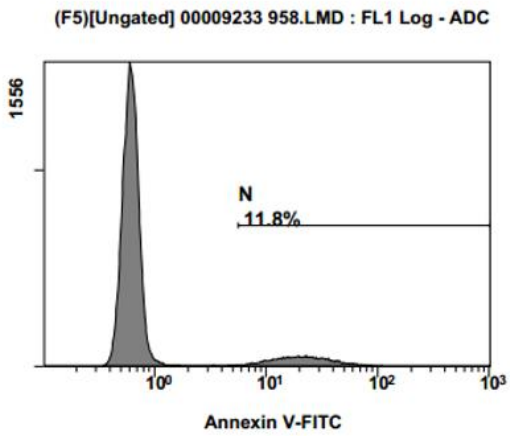
B)



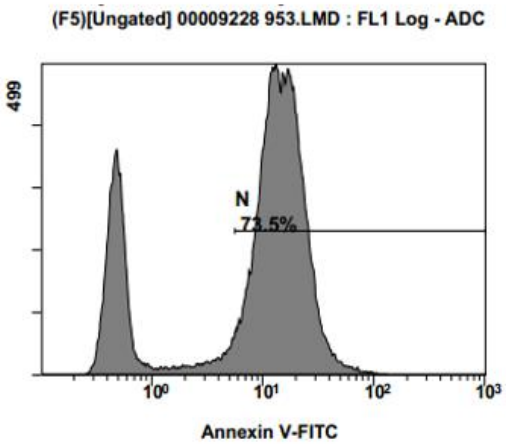
Appendix C Flow cytometry data show early and late apoptotic cells

	- Gln				+ Gln			
	Viable	Early Apoptotic	Late Apoptotic	Necrotic	Viable	Early Apoptotic	Late Apoptotic	Necrotic
AA	55.8±10.2	5.3± 6	38.7±11.2	0.1± 0.1	89.0±1.2	0.3±0.5	9.4±2.2	0.1±0.2
AC	48.4±8.5	4.7±5.7	46.8±11.1	0.0±0.1	87.7±1.5	0.6±0.7	10.0±2.4	0.2±0.3
SA	34.5±8.1	9.1±8.8	56.3±10.9	0.0±0.0	88.3±1.4	0.7±0.8	9.3±1.4	0.0±0.0
SC	29.5±8.1	7.2±8.9	63.2±10.6	0.0±0.0	87.5±1.2	0.5±0.7	10.3±2.2	0.03±0.0
PBS	29.6±8.0	8.9±11.3	46.8±12.1	0.0±0.0	86.7±2.5	0.5±0.9	10.3±1.5	0.0±0.0

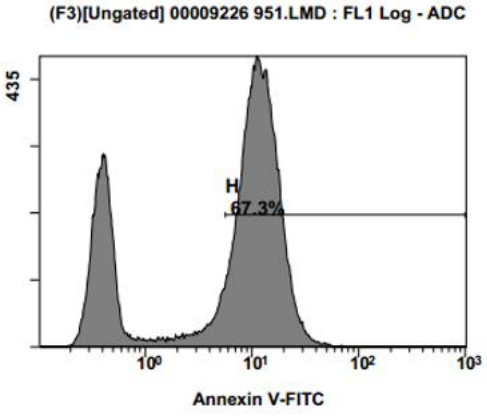
Appendix D A representation of Flow cytometry data (Figure 3.12)



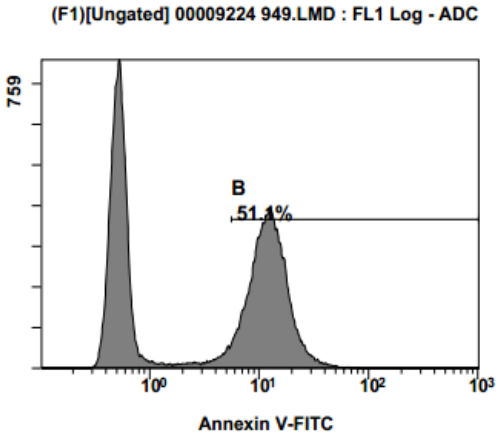
4mM Gln



PBS

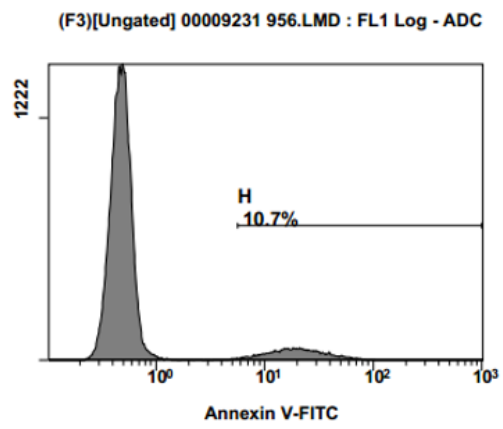


SA

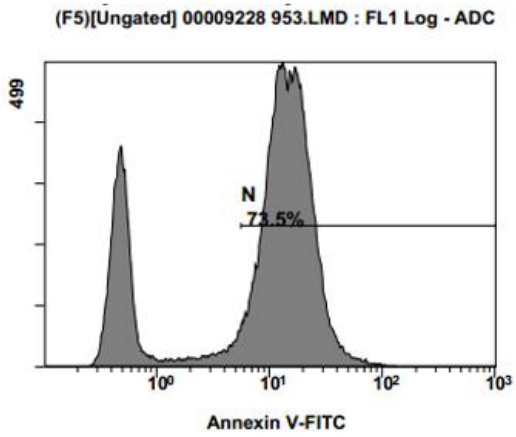


AA

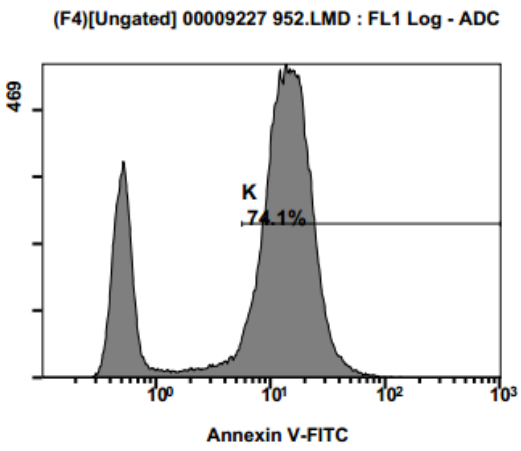
Appendix E A representation of Flow cytometry data (Figure 3.13)



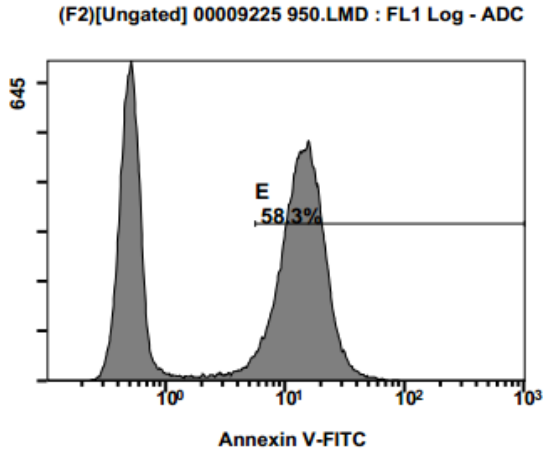
4mM Gln



PBS



SC



AC

Appendix F Densitometry for DON Western blot experiment (Figure 3.24)

Values of Western blot bands are expressed relative to the loading control, Coomassie Blue.

B)

Sample	DON/CASP3 (-Gln)		DON (-Gln)	PBS (-Gln)
	AA	SA		
1	1.2	0.96	1.0	1.2
2	2.5	2.8	2.8	3.0
3	0.9	1.2	1.2	1.2

Sample	DON/CASP3 (-Gln)		DON (-Gln)	PBS (-Gln)
	AC	SC		
1	1.1	1.0	0.8	0.8
2	3.1	3.3	4.0	3.2
3	5.0	6.4	5.0	7.4

C)

Sample	DON/LAMIN (-Gln)		DON (-Gln)	PBS (-Gln)
	AA	SA		
1	3.1	3.4	4.0	3.7
2	3.2	7.0	7.1	8.1
3	0.3	0.6	0.5	1.0

Sample	DON/LAMIN (-Gln)		DON (-Gln)	PBS (-Gln)
	AC	SC		
1	2.2	2.4	2.3	2.5
2	2.3	2.2	2.0	2.3
3	2.9	3.0	2.8	3.1

Appendix G Densitometry for AZA Western blot experiment (Figure 3.25)

Values of Western blot bands are expressed relative to the loading control, Coomassie Blue.

B)

Sample	AZA/CASP3 (-Gln)		AZA (-Gln)	PBS (-Gln)
	AA	SA		
1	1.7	2.3	2.0	2.4
2	1.0	1.2	1.5	1.5
3	4.6	4.5	4.2	4.9

Sample	AZA/CASP3 (-Gln)		AZA (-Gln)	PBS (-Gln)
	AC	SC		
1	1.6	1.9	1.1	1.1
2	0.94	0.8	0.6	0.8
3	2.0	4.9	5.7	4.1

C)

Sample	AZA/LAMIN (-Gln)		AZA (-Gln)	PBS (-Gln)
	AA	SA		
1	4.4	5.0	4.9	5.5
2	3.4	4.2	4.4	4.4
3	3.6	6.5	5.6	6.1

Sample	AZA/LAMIN (-Gln)		AZA (-Gln)	PBS (-Gln)
	AC	SC		
1	3.0	3.2	3.3	3.2
2	2.7	2.2	2.2	2.2
3	3.9	4.6	5.0	7.5